Identification and characterization of mycorrhiza-specific genes in the model plant *Medicago truncatula*

Von der
naturwissenschaftlichen Fakultät
der Universität Hannover
zur Erlangung des Grades
einer Doktorin der Naturwissenschaften
Dr. rer. nat.

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Dipl.-Biol. Jasmin Doll

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Referenten:

PD Dr. Franziska Krajinski

HD Dr. Bernd Huchzermeyer

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Abstract

Medicago truncatula is related to a number of important crop legumes. Unlike many other economically important crop plants, *M. truncatula* can serve as a model system for genetic studies. We have chosen the model plant *M. truncatula* to study the molecular background of the arbuscular mycorrhiza (AM) symbiosis.

With the suppression subtractive hybridization (SSH) technique a cDNA library of exclusively mycorrhiza induced genes of *M. truncatula* has been established. With this technique several transcripts of our model plant were identified showing a strong RNA accumulation in mycorrhizal *M. truncatula* roots, but were not detectable in sterile roots or in roots infected with *Sinorhizobium meliloti.* They were neither detectable after infection with the pathogen *Aphanomyces euteiches* or after treatment with additional phosphate fertilization. Two candidate genes have been selected for further more detailed analyses.

One of these genes, *MtGlp*1, showed high homology to germin-like proteins (GLPs), the second gene encodes for a putative glutathione-S-transferase (GST) and was therefore called *MtGst*1. Members of both gene families are considered to be involved in plant defense and stress response.

In situ hybridization showed a specific expression of *MtGlp*1 in arbuscule-containing cells of *Glomus intraradices* colonized roots of *M. truncatula* and *Lotus japonicus*, indicating that the gene regulation is conserved during AM-colonization of roots in different plant species. Since there is little known about the molecular mechanisms leading to AM-specific transcription activation, promoter studies were carried out with deletion studies of the *MtGst*1 promoter. In addition with EMSA approaches and computational analyses, putative transcription factor-binding sites were detected. A PTGS approach with whole transgenic *M. truncatula* plants was initiated for both genes, giving first results of silencing effects. These transgenic PTGS plants will provide the starting point to unravel the function and influence of these genes during an AM-interaction.

Keywords: arbuscular mycorrhiza, *Medicago truncatula,* Glutathione-S-transferase, Germin-like protein, promoter, Posttranscriptional gene silencing

Zusammenfassung

Die Leguminose *Medicago truncatula* ist mit einer Vielzahl wichtiger ökonomischer Kulturpflanzen verwandt, kann jedoch im Gegensatz zu diesen als Modellsystem für genetische Untersuchungen herangezogen werden. Um Aufschluss über den molekularen Hintergrund der Arbuskulären Mykorrhiza (AM) Symbiose zu erhalten, haben wir uns für die Modellpflanze *M. truncatula* entschieden.

Mit der suppressiven subtraktiven Hybrdisierungstechnik (SSH) wurde eine cDNA-Bank erstellt, die ausschließlich aus mykorrhiza-induzierten Genen von *M. truncatula* besteht. Mit dieser Technik konnten verschiedene Transkripte der Modellpflanze identifiziert werden, die eine starke RNA Akkumulierung in mykorrhizierten Wurzeln aufwiesen, jedoch nicht in sterilen Wurzeln oder mit *Sinorhizobium meliloti* oder mit dem Pathogen *Aphanomyces euteiches* infizierte Wurzeln, auch nicht durch zusätzliche Phosphatdüngung. Zwei viel versprechende Kandidaten wurden ausgewählt, um weiterführende Studien durchzuführen.

Eines dieser Gene, *MtGlp*1, zeigte eine hohe Homologie zu Germin-ähnlichen Proteinen. Die andere Gensequenz codiert für eine Glutathione-S-transferase und wurde *MtGst*1 genannt. Mitglieder dieser beiden Genfamilien scheinen in der Pflanzenabwehr und in Stressantworten involviert zu sein.

In situ Hybridisierung konnte eine spezifische Expression von *MtGlp*1 in Arbuskel-haltigen Zellen mykorrhizierter *M. truncatula* und *Lotus japonicus* Wurzeln detektieren. Dies weißt auf eine konservierte Genregulation in einer AM in verschiedenen Pflanzen hin. Um Aufschluss über molekulare Mechanismen der AM-spezifischen Genregulation zu erhalten, wurden Promoterstudien durchgeführt. Deletionsstudien des Promoters von *MtGst*1, sowie EMSAs und computerbasierende Analysen konnten mögliche Bindestellen für Transkriptionsfaktoren aufzeigen. Ein PTGS Ansatz mit transgenen *M. truncatula* Pflanzen wurde für beide Gene initiiert. Hierfür konnten bereits erste *Silencing* Effekte gezeigt werden. Die transgenen PTGS Pflanzen stellen den Ausgangspunkt dar, um die Funktion und den Einfluss dieser Gene auf das Expressionsmuster anderer Gene in der AM-Interaktion aufzuklären.

Schlagwörter: Arbuskuläre Mykorrhiza, *Medicago truncatula*, Glutathion-S-Transferase, Germin-ähnliches Protein, Promotor, Posttranskriptionelles Gensilencing

Contents

Abbreviations

AM	Arbuscular mycorrhiza
Amp	Ampicillin
AP	Alkaline phosphatase
BAC	Bacterial artificial chromosome
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DNA	Desoxy ribonucleic acid
DNase	Deoxyribonuclease
dsRNA	Double-stranded RNA
dpi	Days post inoculation
EDTA	Ethylene diamine tetra acetic acid
EMSA	Electrophoretic mobility shift assay
EtBr	Ethidiumbromide
EtOH	Ethanol
EST	Expressed sequence tag
for	Forward
GLP	Germin like protein
GST	Glutathione-S-transferase
GUS	ß-Glucoronidase
IPTG	Isopropyl-ß-D-thiogalactate pyranoside
kb	Kilo bases
LB	Luria-Bertani
min	Minutes
MOPS	3-Morpholino-1-propansulfonic acid
NCBI	National Center of Biotechnology Information
OD	Optical density
ON	Over night
OxO	Oxalate oxidase
PAGE	Polyacrylamide gel electrophoresis
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PTGS	Post transcriptional gene silencing

RACE	Rapid amplification of cDNA ends
rev	reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
rpm	Round per minute
RT	Real Time
RT	Reverse transcription
RT	Room temperature
SOD	Superoxide dismutase
SSH	Suppression Subtractive Hybridization
TAE	Tris acetate EDTA
TC	Tentative consensus
Taq	*Thermus aquaticus*
UV	Ultra violet
v	Volume
w	Weight
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D- galactopyranoside

Chapter 1

General Introduction

Legumes

Legumes (*Leguminosae*/ *Fabaceae*) constitute the third largest family of flowering plants with more than 20.000 species (Doyle, 2001; Polhill and Raven, 1981). *Fabaceae* are a highly diverse family and can be split into three subfamilies: *Papilionoideae*, *Caesalpinioideae* and *Mimosoideae* (Doyle and Luckow, 2003). *Papilionoideae* form the largest group representing almost all economically important crop legumes, including soybean (*Glycine max*), peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and alfalfa (*Medicago sativa*). Despite their close phylogenetic relationship, crop legumes differ greatly in their genome size, chromosome number, ploidy level and self-compatibility (http://www.rbgkew.org.uk).

Many legumes are of worldwide agronomic importance, accounting for about 27% of the world's crop production. Economically, legumes represent the second most important family of crop plants after *Pocaceae* (grasses) (Graham and Vance, 2003).

Legumes are of particular value for human and animal nutrition due to their high content of proteins and nitrogen in their seeds and fruits (Cook, 1999). Legumes contribute around one-third of humankind's protein intake. They accumulate secondary metabolites that are beneficial to human health through anti-cancer and other health-promoting activities. Moreover, legumes serve as an important source of fodder and forage for animals and of edible and industrial oils (Dixon and Sumner, 2003). Most economically important legume plants are not suitable for a model system for genetic studies, above all, because of their large genome size and their complexity (Frugoli and Harris, 2001).

The model legume *Medicago truncatula*

M. truncatula is originally grown in Mediterranean areas, South Africa and Australia

(Crawford *et al.*, 1989) and is classified as a legume plant. *M. truncatula*, also known as barrel medic, is a close relative of the world's most important forage legume, alfalfa (*Medicago sativa*). It was shown that the genomes of the two *Medicago* species are essentially co-linear (Choi *et al.*, 2004). Furthermore, in addition to alfalfa, *M. truncatula* is related to a number of important crop legumes such as pea, chick pea, faba bean, lentil and clover (Doyle *et al.*, 1996). Unlike many other major crop legumes, *M. truncatula* can serve as a model legume. In comparison, *M. truncatula* has a genome size of around 500 - 600 Mbp, which is only half of alfalfa, but almost 4 times larger than that of *Arabidopsis thaliana*. Still the genome of *M. truncatula* is almost 10 times smaller than that of pea (Blondon *et al.*, 1994).

M. truncatula stands above other plants as a model for molecular genetic research because for a variety of reasons. *M. truncatula* is an annual diploid plant (2 n = 16). Alfalfa is tetraploid and therefore not suitable for genomic research. Moreover, *M. truncatula* is self-fertile, whereas alfalfa is an obligate, out-crossing species. The short growth, prolific seed production and efficient methods of transformation have made it the system of choice for many studies of basic and applied legume research. High levels of macro- and microsyntheny of *M. truncatula* to pea, alfalfa and soybean were identified (Gualtieri *et al.*, 2002; Thoquet *et al.*, 2002). This suggests, that the genomic information obtained by research with *M. truncatula,* could be easily transferred to other legume crops, such as pea, faba bean, alfalfa, lentils, clover and chick pea.

In addition to genomic studies with the most studied genetic model plant *A. thaliana, M. truncatula* emerged as a model system for legume biology in the 1990s (Barker *et al.*, 1990; Bell *et al.*, 2001; Cook, 1999). Since then, the resources for this system have expanded and a number of interesting characteristics for both molecular and classical genetics exist (Barker *et al.*, 1990; Cook, 1999).

The recent completion of the *Arabidopsis* genome sequencing project has provided enormous data for all plants. Yet a very low level of microsyntheny between *M. truncatula* and *A. thaliana* exists. This suggests that we can not transfer the knowledge resulting from the *Arabidopsis* genome to legume genomes in general (Harrsion, 2000).

Large genome sequencing projects for *M. truncatula* have been initiated (*Medicago truncatula* Gene Index, MtGI) and the database is available to the public (www.medicago.org; www.genome.ou.edu/medicago.html). Up to now, more than 200,000 ESTs were generated from more than 30 different cDNA libraries. These

libraries contain developmental stages in different plant organs and those that are expressed under a range of physiological conditions, stresses, as well as abiotic and biotic interactions (Frugoli and Harris, 2001). Corresponding microarray and DNA chips are currently available. Gene knockout systems, RNA interference and gene tilling are under development (Young *et al.*, 2005).

Unlike the most studied genetic model plant, *A. thaliana*, *M. truncatula* establishes symbiotic relationships. *M. truncatula* is able to form complex interactions with atmospheric nitrogen-fixing symbiotic bacteria. Furthermore, these plants are able to form symbiotic root mycorrhizae with soil borne fungi (Gianinazzi-Pearson, 1996). During the last several years, a large variety of *M. truncatula* ecotypes has been described and genetically characterized. These derive from different Mediterranean habitats representing a wide adaptation to diverse living conditions (Jenczewski *et al.*, 1997; Prosperi, 2000; Prosperi *et al.*, 2001). Due to the natural genetic diversity and the powerful genetic tools available for *M. truncatula* research, this plant has become a popular model for investigating plant-microbe interactions.

Mycorrhiza symbiosis

In the 1880s, the German biologist A. Frank had found fungi becoming interconnected to the plants by a mycelium network near plant roots. But to his astonishment, the plants did not show any disease. He named this symbiosis mycorrhiza, derived from the Greek *mykes* for fungus and *rhiza* for root. Frank surmised that this association was based on bi-directional nutrient exchange (Frank, 1885).

Mycorrhizas are symbiotic relationships formed between fungi and plant roots. More than 90% of terrestrial plants are associated with root-colonizing mycorrhiza fungi.

Different types of mycorrhiza exist, each of them representing a distinct type of association. Some kinds of orchids are also capable of forming a mutualistic mycorrhiza symbiosis (Read *et al.* 2000). However, the two major forms of mycorrhiza are the endotrophic mycorrhiza, known as arbuscular mycorrhiza (AM), and the ectrotrophic mycorrhiza. Ectomycorrhizas are characteristic for many trees, such as pines, firs, oaks, birches in the northern hemisphere and eucalypts in Australia. This interaction is formed between the tree roots and fungi of the orders *Basidiomyceta*, *Ascomycta* and *Zygomyceta* (Wiempken and Boller, 2002). Arbuscular mycorrhizas are the most widespread type of mycorrhizal association. AMs exist in ecosystems all over the world

where they create an intimate link between plants and the rhizosphere (Smith and Read, 1997).

The association between plant roots and fungi is characterized by a bi-directional exchange of nutrients improving the living conditions for both the fungal partner and the plant. Colonization of the plant roots with mycorrhiza fungi can greatly improve plant growth and yield. Furthermore, mycorrhizal plants are more resistant against abiotic or biotic stress conditions (Augé, 2001), exhibiting a better protection against plant pathogenic attack (Cordier *et al.*, 1996; Slezack *et al.*, 2000).

Arbuscular mycorrhiza

More than 400 million years ago, in the Devonian era, the symbiotic interaction between fungi and plant roots developed coevolutive strategies to improve their living conditions. Hypotheses suggest that the formation of an interaction between fungi and plants might be responsible for the adaptation of plants to the land (Pirozynski and Dalpe, 1989; Simon *et al.* 1993). Current observations of fossil records exposed the association of AM-fungi together with the root system of plants since they first colonized land (Remy *et al.*, 1994). Certainly, it is clear that the ability to form an AM-interaction occurred early in the evolution of plants.

Today, the capacity to form this association is distributed widely throughout the plant kingdom. More than 80% of all vascular plants engage in AM-associations (Newman and Redell, 1987). Only a few plant species, such as *Brassicaceae*, *Caryophyllaceae*, *Chenopodiaceae* or *Urticaceae* are not capable of forming an arbuscular mycorrhiza (Smith and Read, 1997).

The AM-fungi belong to the order *Glomeromycota* (Schüssler *et al.*, 2001). They are obligatory biotrophic, establishing a symbiotic relationship in order to obtain carbon, which enables them to grow and complete their life cycle (Smith *et al.,* 2003). So far, AM-fungi have not been cultured in the absence of a plant host and therefore relatively little is known about them.

Development of an AM-interaction

When not in association with plant roots, AM-fungi rest in the soil as spores. In many

plant-microbe symbioses, detection of the corresponding partner happens prior to direct contact. In some instances a molecular dialog initiates events essential for progression to the physical stages of the interaction. Germination of AM-fungal spores can occur in the absence of plant roots. Before infection, AM-fungi recognize and respond to their potential hosts. Root exudates elicit rapid and extensive branching of hyphae as they come close to neighboring roots (Giovannetti *et al.*, 1996).

Development of the symbiosis is initiated when fungal hyphae come in contact with the root of a host plant. Upon contact, the topographical and biochemical properties of host root epidermal cell walls induce the formation of AM-fungal appressoria on cortical or vascular cell walls (Giovannetti *et al.*, 1993; Nagahashi and Douds, 1997). Starting from the appressoria, the fungus penetrates the root surface and colonizes the intercellular space of the root cortex (Perotto and Bonfante, 1997). Hyphae penetrate the cell walls and develop specific structures within the cortex cells. The internal development of the fungus is influenced by the plant.

Different morphological growth patterns depending on the plant partner in the association exist. The two main patterns are termed as *Paris* and *Arum* types, named after the species in which they were first described (Gallaud, 1905). In *Arum* type, fungal hyphae spread between cortical cells and form short-lived heavily branched symbiotic structures within root cells. These highly branched haustorium-like structures, called arbuscules, give this type of symbiosis its name. The characteristic of the *Paris* type is the intracellular colonization of the root cortex with thick coiled hyphae, which occasionally form fine arbuscule-like ramifications.

Bi-directional nutrient exchange

All mutualistic plant-microbe interactions show the same trading patterns and similar structural features. The plant supplies carbohydrates to the fungus whereas the fungus assists the plant with the acquisition of phosphate and other mineral nutrients from the soil (Harrison, 1998). Nutrient transport between symbionts is a central aspect of the symbiosis. The beneficial effect of the AM-symbiosis results from a complex molecular dialog between the two symbiotic partners (Harrison, 1999). In terms of the transport of individual nutrients, the transfer of carbohydrates is thought to be the main benefit for the fungal symbiotic partner (Pfeffer *et al.*, 1999).

The arbuscules, presenting a large area of close contact between the symbionts, were traditionally assumed to be the interface through which the carbon transfer takes place. However, the observation that the arbuscule membrane lacks ATPase activity suggests that carbon uptake might not occur via the arbuscules. High ATPase activity has been observed in the intercellular hyphae. This led to the suggestion that membranes of these hyphae played an important role in active transport processes (Smith *et al.*, 1993, Harrison, 1999).
The nutrient exchange may be of critical importance when soil fertility and water availability are low, conditions that severely limit agricultural production in many parts of the world.

Functional genomics - a tool to study symbiotic interactions

The sequencing project of the human genome represents one of the largest challenges in the history of science. Also a large number of sequencing projects for several model organisms are in progress or already completed, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *A. thaliana.* The information, gathered from these projects, provide a lot of raw data to explode the field of bioinformatics and to investigate in molecular biological approaches. Genomics is now undergoing a change, providing a broad range of new projects. To reflect this diversification, genome analyses were divided into „structural genomics“, „comparative genomics” and „functional genomics“. „Structural genomics” will provide genetic and physical maps to arrange the localization of important genes on the genome. „Comparative genomics“ focuses on the correlation of genetic information received from one model plant to the corresponding plant family e.g. crop plants of economic importance which can not be used as model plants. With „functional genomic” approaches, a better understanding of gene expression and gene function will be achieved (Hieter and Boguski, 1997).
Recently, the first insight into molecular mechanisms underlying the development of the AM-symbiosis has been obtained. Responsible specifically regulated genes could have been identified. Functional genomic approaches may be a tool to gain a better appreciation of the function of these genes and their role in plant-microbe interactions. This will allow us to gain a better understanding of the molecular processes driving an AM-interaction.

The aim of this work

Transcription profiles of *M. truncatula* during an AM were arranged in order to gain important new insight into molecular and physiological changes during the interaction. Recently, transcription profiling has become synonymous for gene expression analysis. A technique called suppression subtractive hybridization (SSH) has been used to compare gene expression patterns in different gene populations identifying exclusively expressed genes in mycorrhizal *Medicago* roots. As a result, rare differentially expressed transcripts can be enriched more than 1000 fold (Diatchenko *et al*., 1996). By this method, we identified a set of AM-specifically induced genes of the model plant *M. truncatula*. Two candidate genes have been selected for further more detailed analyses, including the localization of the AM-specific gene transcripts, the regulation process and their function in the interaction. The analyses presented in this work focus on four major objectives:

– Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*.
– A member of the germin-like protein family is a highly conserved mycorrhiza-specific induced gene.
– Insights into the transcriptional regulation of *MtGst*1 - an AM-specific gene of *Medicago truncatula*.
– PTGS approach to unravel the function of *MtGlp*1 and *MtGst*1, two AM-specific *Medicago truncatula* genes.

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Chapter 2

Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*.*

Wulf A, Manthey K, **Doll J**, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Kuester H, Krajinski F

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Transcriptional Changes in Response to Arbuscular Mycorrhiza Development in the Model Plant *Medicago truncatula*

Anne Wulf,[1] Katja Manthey,[2] Jasmin Doll,[1] Andreas M. Perlick,[2] Burkhard Linke,[3] Thomas Bekel,[3] Folker Meyer,[3] Philipp Franken,[4] Helge Küster,[2] and Franziska Krajinski[1]

[1]Department of Molecular Genetics, University Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany; [2]Department of Genetics, University Bielefeld, 33594 Bielefeld; Germany; [3]Center for Genome Research, University Bielefeld, 33594 Bielefeld; Germany; [4]Institute for Vegetables and Ornamental Plants, Theodor-Echtermeyer-Weg, 14979 Grossbeeren, Germany

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Significant changes in root morphology and physiology during arbuscular mycorrhiza (AM) development are likely to be controlled by specific gene expression pattern in the host plant. Until now, little was known about transcriptional changes which occur AM-exclusively; that is, they do not occur during other root–microbe associations, nor are they induced by improved phosphate nutrition. In order to identify such AM-exclusive gene inductions of *Medicago truncatula*, we used a pool of different RNA samples as subtractor population in a suppressive subtractive hybridization (SSH) experiment. This approach resulted in the identification of a number of new AM-regulated genes. None of these genes were expressed in nonmycorrhiza roots or leaves. Electronic data obtained by comparison of the cDNA sequences to expressed sequence tag (EST) sequences from a wide range of cDNA libraries in the *M. truncatula* EST database (Gene Index, MtGI) support the mycorrhiza specificity of the corresponding genes, because sequences in the MtGI that were found to match the identified SSH-cDNA sequences originated exclusively from AM cDNA libraries. The promoter of one of those genes, *MtGst*1, showing similarities to plant glutathione-S-transferase (GST) encoding genes, was cloned and used in reporter gene studies. In contrast to studies with the potato GST gene PRP, *MtGst* 1 promoter activity was detected in all zones of the root cortex colonized by *Glomus intraradices*, but nowhere else.

Additional keywords: quantitative real-time RT-PCR.

Legumes have the unique capacity among terrestrial plant species to form two different symbioses. They can interact with the nitrogen-fixing *Rhizobium* spp. and, in addition, with arbuscular mycorrhiza (AM) fungi that provide the host plant with phosphorous and other soil nutrients (Smith and Read 1997). In contrast to the well-understood legume–*Rhizobium* symbiosis, the understanding of molecular regulation underlying AM development is limited. One reason for this phenomenon is probably the obligate biotrophic nature of the AM fungi. Together with the fact that AM fungi are asexual, multinucleate organisms, this makes it very difficult to study gene regulations of the microsymbiont in AM (Franken and Requena 2001; Kuhn et al. 2001). In contrast, rapidly increasing data obtained after establishment of genome research programs for model plants capable of forming AM provide new opportunities for detailed insights into plant gene regulation during AM formation.

Development of the tight association between plant roots and AM fungi is supposed to involve a complex network of signal perception, amplification, and transduction in the host plant (Harrison 1999). Highly complex new structures, arbuscules, are formed inside root cortical cells and an AM is characterized by an unique physiological condition; therefore, it is likely that the symbiosis is controlled by AM-exclusive gene regulations in the host plant. In recent years, extensive efforts have been made using nontargeted approaches such differential RNA display or suppressive subtractive hybridization (SSH) to identify plant genes involved in AM symbiosis (Lapopin and Franken 2001). Some of the identified mechanisms were found to be conserved with those of the *Rhizobium*–legume symbiosis, suggesting that the two plant-microbe interactions share common signal transduction pathways (Hirsch and Kapulnik 1998; Staehelin et al. 2001; van Rhijn et al. 1997). The recent identification of a receptor-like protein kinase, which is required for entering the AM symbiosis and the nitrogen-fixing root nodule symbioses, corroborates the belief in partially overlapping genetic programs in both symbioses (Endre et al. 2002; Stracke et al. 2002). Furthermore, the large majority of AM-regulated plant genes identified so far turned out to be regulated in a nonspecific way, because they are not exclusively regulated by AM. For example, some AM-induced genes turned out to be induced also during pathogenic interactions (Krajinski et al. 1998, Lapopin et al. 1999) or by increased phosphate nutrition (Burleigh and Harrison 1997). Nevertheless, particularly with regard to arbuscule development and unique AM physiology, it is likely that specific mechanisms control AM development; however, up to now, only a few examples exist for AM-specific genes (Salzer et al. 2000). Therefore, our aim was to identify transcriptional changes in the host plant which occur exclusively, in terms of other plant-microbe interaction or phosphate nutrition, in response to AM development. *Medicago truncatula* is currently the subject of major plant genome research programs (Barker et al. 1990; Bell et al. 2001; Cook 1999; Frugoli and Harris 2001; Oldroyd and

Corresponding author: F. Krajinski,
E-mail: krajinski@lgm.uni-hannover.de

Sequence data of the *MtGst* 1 gene and promoter can be found at GenBank as accession numbers AYB4608 and AYB43609. Mtgmacc-EST data can be found at AJ499169 to AJ500943.

Geurts 2001). Thus, to gain information on mycorrhiza-specific gene expression, we exploited the potentials of modern genome research using *M. truncatula* as a model plant.

RESULTS

Characterization of *M. truncatula–Glomus intraradices* mycorrhizae.

M. truncatula seedlings were inoculated with *Glomus intraradices* and harvested 3 weeks after inoculation. At this point, the frequency of infection (*F*) was nearly 100%, the colonization intensity (*M*) was 81%, and all developmental stages of AM, appressoria, arbuscules (relative arbuscule frequency *a* = 81%), intercellular hyphae, and extraradical hyphae were observed. This material was used to analyze AM-induced gene expression.

Cloning of AM-specifically induced genes using SSH.

A subtracted cDNA library enriched with *M. truncatula* cDNA sequences that correspond to mycorrhiza-specifically transcribed genes was generated by SSH (Diatchenko et al. 1996). In order to obtain genes, which are mycorrhiza-specifically induced, mycorrhizal-root cDNA was subtracted by a cDNA mixture of plants obtained from four different treatments. Amongst those were plant roots treated with 5 mM phosphate to avoid cloning of genes, which are induced by the improved phosphate nutrition. For excluding genes, which are also induced during pathogenic interaction or during nodulation, *M. truncatula* plants were infected with an oomycete pathogen, *Aphanomyces euteiches,* or with *Sinorhizobium meliloti*. A fourth set of nonmycorrhiza plants were grown without any interacting organisms. All plants were harvested 3 weeks after inoculation (Fig. 1).

The resulting SSH cDNA fragments were cloned and a total number of approximately 2,000 clones were obtained. Insert sizes varied from 300 to over 1,500 bp and the average was 600 bp (excluding primer sequences).

Analysis of SSH cDNA sequences.

Sequencing of the SSH cDNA fragments resulted in 1,805 expressed sequence tag (EST) sequences (accession numbers AJ499169 to AJ500943). SSH-cDNA sequences were designated as *M. truncatula–G. intraradices* mycorrhiza (*MtGim*) ESTs. Clustering according to the TIGR *M. truncatula* gene index protocol (Liang et al. 2000) resulted in 600 singletons and 290 tentative consensus (TC) sequences. In all, 55 of the TC sequences and 125 of the singleton sequences with sequence length of >100 bp did not show any significant similarities (below $1e^{-10}$) to ESTs deposited in the *M. truncatula* gene index. Of this 180 sequences which do not match any already existing *M. truncatula* ESTs, 15 TC sequences and 38 singletons showed significant homologies to genes after blastx-search. TC number 167, which was assembled from 55 SSH ESTs, was the most redundant sequence among the 1,805 ESTs. The corresponding cDNA is identical to the recently described *MtPT*4 gene, a phosphate transporter which is involved in the plant phosphate uptake in arbuscule-containing cells (Harrison et al. 2002).

In a first attempt to analyze this library, cDNA inserts of 34 clones were amplified and analyzed by reverse Northern hybridizations. Two sets of membranes were prepared: one was hybridized with labeled cDNA synthesized from mycorrhizal roots, and the other with labeled cDNA of the nonmycorrhiza RNA pool extracted from control roots, phosphate-fertilized roots, and roots infected by *A. euteiches* or *S. meliloti*. The housekeeping gene *MtEf1-a*, encoding the translation elongation factor EF-1 α, was used as a nonregulated control gene and the same amounts of the corresponding cDNA were added to each membrane. The *MtEf-1a* cDNA showed even and strong hybridization to nonmycorrhiza pool cDNA and to mycorrhiza cDNA, respectively. In contrast, 22 of the 34 inserts showed clearly increased RNA accumulation after hybridization to the mycorrhiza cDNA probe, indicating AM-induced RNA accumulation of the corresponding genes (Table 1).

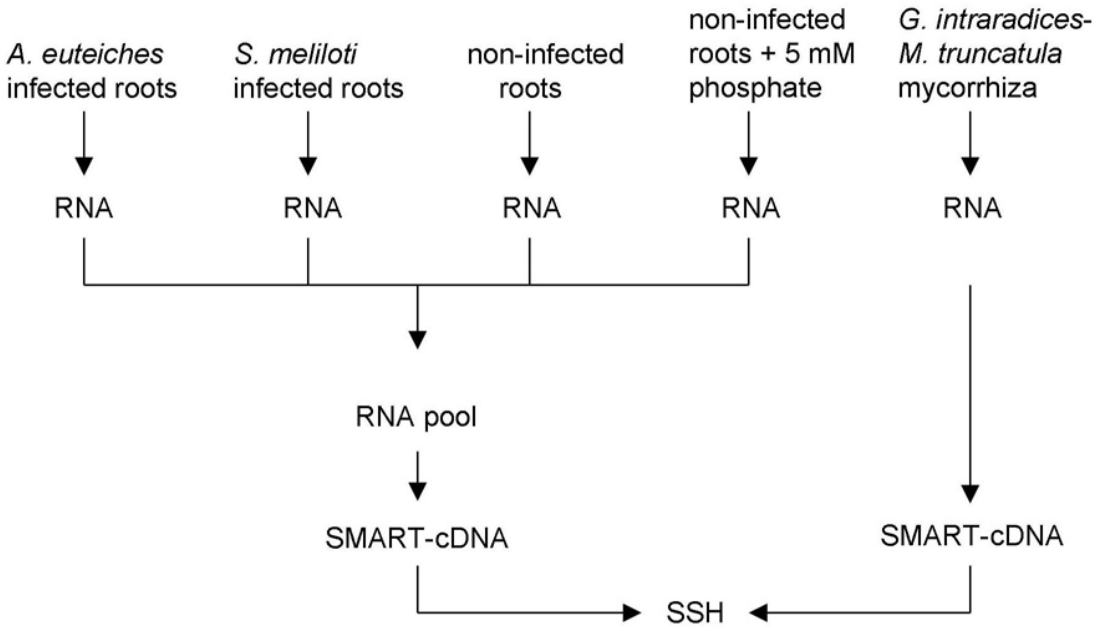

Fig. 1. Flowchart of the experimental design to obtain a cDNA library enriched for genes specifically induced in the mycorrhiza of *Medicago truncatula*. Seedlings of *M. truncatula* (3-day-old) were inoculated with *Glomus intraradices*, *Sinorhizobium meliloti*, or *Aphanomyces euteiches*. All plants were fertilized with half strengths Hoagland solution, half of the noninfected control plants obtained increased amounts (5mM) phosphate. RNA was extracted of the roots three weeks after inoculations. Equal amounts of RNA of all treatments except mycorrhiza were sampled in one pool. SMART-cDNA was prepared and used for suppression subtractive hybridization (SSH).

Analysis of the cDNA sequences showed that 20 mycorrhiza-induced genes were identified, of which 11 revealed significant similarities to different plant genes on the amino acid level. Comparison of these 20 *MtGim*-cDNAs to the *M. truncatula* gene index showed that all *M. truncatula* TC sequences that were found to match these *MtGim* cDNAs cover ESTs originating exclusively from AM cDNA libraries. Hence, this in silico expression analysis further supports a mycorrhiza-specific gene induction.

Three different sequences with similarities to plant lectins were among the induced cDNAs (*MtGim* 3, 11, and 14). *MtGim* 27 encodes a part of the *MtPT*4 phosphate transporter gene (Harrison et al. 2002) which was strongly induced after hybridization to the AM cDNA. In addition to this phosphate transporter, we found a second transporter-encoding gene. One fragment, *MtGim* 7, encodes a putative nitrate transporter. Another strongly AM-induced fragment, *MtGim* 29, has similarities to plant glutathione-S-transferases (GSTs). Most of the identified genes have not been described to be involved in AM functioning before. *MtGim* 12 has similarities to an *Arabidopsis thaliana* syringomycin biosynthesis enzyme-like protein, and *MtGim* 13 is similar to plant blue copper binding proteins. *MtGim* 31 encodes a germin-like protein and *MtGim* 32 is highly similar to a tissue-specific protein of another legume plant, *Cicer arietinum*. *MtGim* 30 encodes a gene with similarities to miraculin-like proteins, which belong to the superfamily of plant Kunitz-type proteinase inhibitors.

Relative quantification of gene expression by real-time reverse transcriptase-polymerase chain reaction.

Sequences with significant similarities to plant genes not previously described to be involved in AM were selected for further expression analysis. Further analyses of RNA accumulation concerning tissue specificity and gene induction in AM were carried out by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The *MtEf-1a* gene was chosen as active reference control and a sequence encoding part of the glyceraldehde-3-dehydrogenase (*Gapdh*) of *G. intraradices* (Accession number BI452322) was used as a marker for fungal gene expression in mycorrhiza tissues.

A new set of mycorrhiza and noninfected control plants were grown and harvested 3 weeks after inoculation. Similar mycorrhiza parameters compared with the first set of mycorrhiza plants which were used for SSH were observed. RNA was extracted from roots and leaves of control and mycorrhizal plants. All RNA preparations were checked for DNA contaminations by PCR using primer combinations that span intron sequences (data not shown). Total RNA was used as template for one-step RT-PCR and *MtEf-1a* was found to be the most abundant gene showing constant expression among the RNA samples tested. Comparative RNA accumulation levels of the genes represented by the *MtGim* numbers are shown in Figure 2. Melting curves were analyzed after amplification reactions and single amplification products were present in all reactions. All analyzed genes clearly showed induced RNA accumulation in mycorrhiza. No significant RNA accumulation was detectable in leaves of mycorrhiza or nonmycorrhiza plants.

Analysis of *MtGst*1 sequence.

The *M. truncatula* gene encoded by *MtGim* 29 was selected for further analysis. It was one of the most redundant sequences among the 1,805 EST sequences of the SSH cDNA library; 42 ESTs could be aligned to one TC encoding the corresponding cDNA. *MtGim* 29 showed similrities to plant GSTs and clearly was induced in *G. intraradices*-colonized roots. The promoter of the previously described GST-encoding gene, PRP1 of potato, was found to activate transcription in arbuscule-containing cells during AM, as well as during various pathogenic interactions (Strittmatter et al. 1996). This is in contrast to the postulated mycorrhiza-specific expression pattern of *MtGim* 29, because the applied SSH strategy indicates a mycorrhiza-exlusive induction for this gene. To verify mycorrhiza-exclusive induction of *MtGim* 29, RT-PCR experiments were carried out with cDNA extracted from roots

Table 1. Similarities at DNA or protein level between *Medicago truncatula–Glomus intraradices* mycorrhiza (*MtGim*) expressed sequence tags showing increased RNA accumulation in arbuscular mycorrhiza and sequences in databases

MtGim no.	Mtgmacc index	Matching sequence from the GenBank "nr" data base (blastX)[a]	Origin of matching sequence	E value (blastX)	TIGR MtGI index[b]	Induction level[c]
1	2a05	No hits below 1e	…	…	…	+++
3	2b11	Lectin precursor	*Glycine max*	3e-25	AL383972	+++
5	2c11	No hits	…	…	…	+
7	2d08	Putative nitrate transporter	*Oryza sativa*	5e-42	AL383332	+++
9	2d11	No hits below $1e^{-10}$	…	…	…	+
11	2e04	Mannose/glucose-binding lectin CLAII precursor	*Cladrastis lutea*	2e-21	…	+++
12	2e08	Syringomycin biosynthesis enzyme-like protein	*Arabidopsis thaliana*	2e-16	TC56482	++
13	2e09	Blue copper-binding protein-like	*Arabidopsis thaliana*	2e-12	AW584704	+++
14	2e10	Seed lectin	*Dioclea guianensis*	2e-19	AL382121	+++
15	2f05	No hits below $1e^{-10}$	…	…	…	+
22	2h07	No hits	…	…	…	+++
23	3a02	No hits	…	…	…	+
24	3a03	No hits	…	…	…	+++
25	3a04	No hits	…	…	…	+
26	3a05	No hits below $1e^{-10}$	…	…	…	++
27	03b05	Phosphate transporter MtPT4	*Medicago truncatula*	1e-52	TC54414	++
29	1a01	2,4-D inducible glutathione S-transferase, putative	*Arabidopsis thaliana*	1e-19	TC53652	++
30	2h11	Miraculin precursor	*Richadella dulcifica*	4e-07	TC50768	++
31	4e10	Germin (oxalate oxidase)-like protein	*Oryza sativa*	1e-24	TC54411	++
32	3g04	Specific tissue protein	*Cicer arietinum*	4e-46	TC45744	+++
33	…	Elongation factor 1-α	*Lilium longiflorum*	0.0	TC50975	0

[a] Nr = all nonredundant GenBank coding sequences.

[b] Identities in the TIGR *M. truncatula* gene index (MtGI).

[c] Induction levels were classified on the basis of cDNA hybridisation in reverse Northern blot experiments: +++ = very strong signals after hybridization to mycorrhiza cDNA, no signals detectable after hybridization to the nonmycorrhiza cDNA pool; ++ = signals after hybridization to mycorrhiza cDNA, no signals detectable after hybridization to the nonmycorrhiza cDNA pool; + = signals after hybridization to mycorrhiza cDNA but weak signals also detectable after hybridization to the nonmycorrhiza cDNA pool; 0 = no signal difference.

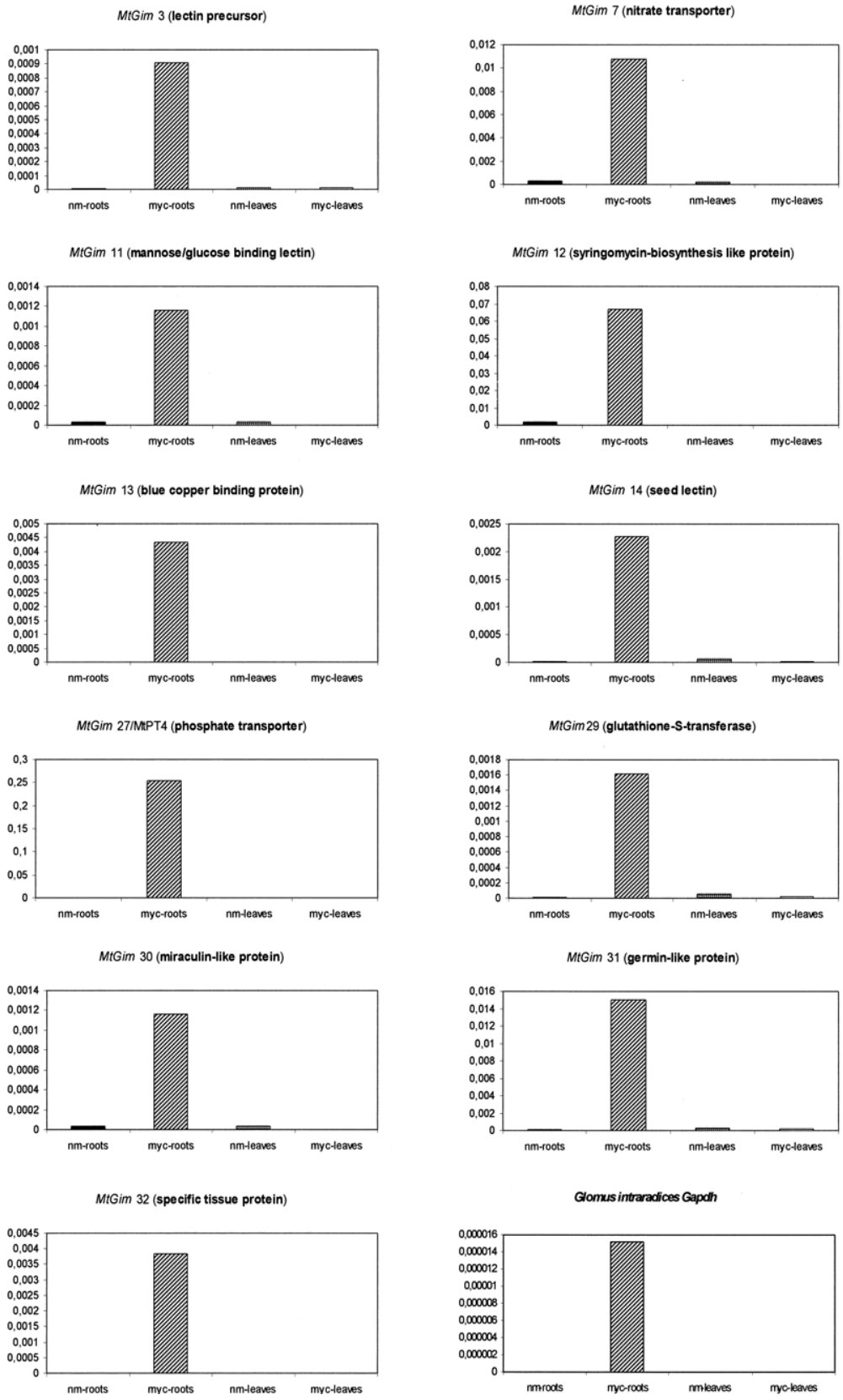

Fig. 2. Graphical representation of comparative expression levels of analyzed genes represented by *MtGim*-EST numbers. Template RNA was extracted from noninfected control roots (nm-roots), mycorrhiza (myc-roots), leaves of noninfected control plants (nm-leaves), leaves of mycorrhizal plants (myc-leaves). Ct-values were determined by quantitative real-time polymerase chain reaction and were used to calculate comparative expression levels ($2^{-\Delta Ct}$). Expression levels are relative to the level of *Mtef*-1α expression, which was constant in all RNA samples used and was set at 1.

of AM, control roots of both phosphate treatments, and roots infected with *S. meliloti* or *A. euteiches*. Using *MtGst*1-specific primers, amplification products were obtained only using AM-cDNA as a template, whereas transcripts of *MtEf*1- *a* transcripts were amplified in equal amounts from each cDNA template tested (Fig. 3), proving an AM-specific transcription of *MtGim* 29 in terms of the analyzed conditions.

Rapid amplification of cDNA ends (RACE) of the 5′ and 3′ ends was carried out, and a 1,034-bp full-size cDNA sequence was obtained, carrying a putative 666-bp open reading frame. The deduced amino acid sequence was 55% identical to a putative 2,4-D inducible glutathione transferase of soybean (Accession number T06239). The corresponding *M. truncatula* gene was named *MtGst*1, according to the proposed nomenclature for *M. truncatula* (VandenBosch and Frugoloi 2001). In order to localize the activiation of the gene, it was attempted to use promoter-reporter constructs in transformed roots, as described by Boisson-Dernier and associates (2001). Inverse PCR (IPCR) was applied to clone the promoter region of *MtGst*1 and a 1,700-bp sequence of the *MtGst*1 upstream region was obtained. A putative TATA box was identified 29 bp from the transcription start.

*MtGst*1 is highly expressed in response to root colonization by *G. intraradices*.

Transgenic *M. truncatula* roots carrying 895 bp of the promoter region in front of the GUS-reporter gene were produced via *Agrobacterium rhizogenes*-mediated transformation. Plants which developed Kanamycin-resistant hairy roots were transferred into substrate containing or not containing inoculum of *G. intraradices*. Three weeks after transplanting, roots were harvestet and analyzed for GUS actvity, then stained with ink to visualize fungal structures which appeared in dark blue.

No GUS activity was observed in roots that were grown in the absense of the AM fungus. Roots that were inoculated with *G. intraradices* were heavily colonized with this fungus; estimation of mycorrhiza parameter showed no difference to the wild-type *M. truncatula-G. intraradices* mycorrhiza. Intense GUS activity was detectable in root parts colonized by the AM fungus but nowhere else. Also, external fungal hyphae did not stain. Root fragments which harbored mycorrhiza showed strong GUS activities in arbuscule-containing regions (Fig. 4), but GUS activity was not limited to the arbuscule-containing cells. Nevertheless, arbuscule-containing cells showed the most intense promoter activity.

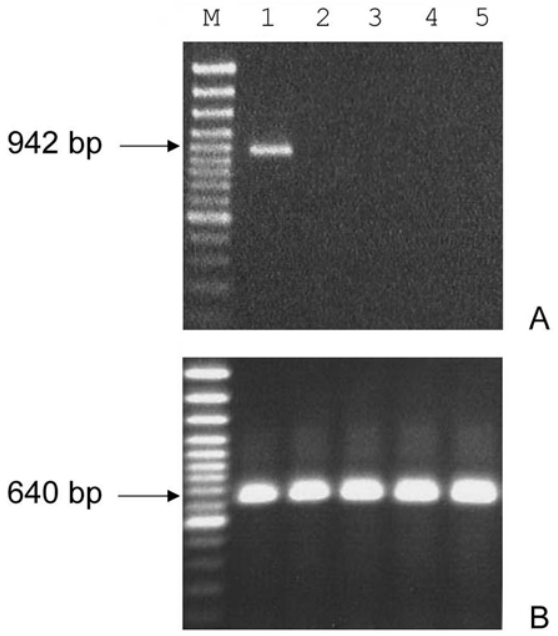

Fig. 3. Reverse transcriptase-polymerase chain reaction analysis of **A,** *MtGst*1-RNA and **B,** *MtEf*1-α RNA accumulation. cDNA was synthesized from RNA of *Medicago truncatula* mycorrhiza (1) or of *Sinorhizobium meliloti* (2) or *Aphanomyces euteiches* (3) infected roots, noninfected roots (4), and noninfected roots grown with increased (5mM) phosphate nutrition and amplified with *MtGst*1 (A) or *MtEf*1-α (B) specific primers. Sizes of amplification products are indicated. M = DNA size marker.

DISCUSSION

Development of the AM is associated with significant alterations in root morphology and physiology of the host plant. Therefore, it is likely that the symbiosis development reqiures a specific and coordinated regulation of gene expression in the host plant. Here we demonstrate that the application of SSH using a pool of different RNA samples as subtractor population is an efficient way to identify mycorrhiza-specific plant genes. The addition of RNA from nodulated and pathogen-infected roots to the subtractor RNA pool resulted in the subtraction of genes, which are induced not exclusively during AM but also during pathogenic associations, during the nitrogen-fixing symbiosis with rhizobia, or by increased phosphate nutrition. This approach resulted in the generation of 1,805 SSH ESTs. From the EST collection, 2.97% matched the cDNA sequence of *MtPT*4, an *M. truncatula* AM-induced phosphate transporter that is transcribed exclusively in arbuscule-containing root cortical cells (Harrison et al. 2002). With regard to phosphate uptake as one key element of an AM, the redundant presence of *MtPT*4 as a marker gene for a functional AM symbiosis in the SSH EST collection indicates that the applied SSH technique succesfully enriched cDNAs of AM-induced genes.

Transcripional changes observed by subtraction and hybridization methods were confirmed by quantitative real-time RT-PCR. Using this technique, we could determine comparative expression levels of AM-specific plant genes and verify the tissue specificity of gene induction. Expression levels were determined relative to the level of *MtEf-1a* expression, which was constant in all template RNA samples and set 1. EF-1*a* is a very abundant protein in eucaryotic cells that catalyzes the binding of tRNA molecules to ribosomes (Slobin 1980). All of the analyzed *M. truncatula* genes showed a much higher RNA accumulation in mycorrhiza roots compared with control root level, but none of them reaches the high level of *Mtef*-1*a*. *MtGim* 27 (*MtPT*4) reaches a relative expression level of 0.254 in mycorrhiza roots, the highest comparative expression level measured in this study.

The expression levels in leaves of mycorrhiza and control plants was determined, because recent studies on PR-protein expression in tobacco suggested the existence of regulatory processes, initiated in the roots of mycorrhiza plants that modify gene expression in their leaves (Shaul et al. 1999). None of the analyzed genes were regulated in leaves, indicating that the analyzed genes do not belong to this kind of regulatory processes.

The mycorrhiza-specific induced sequences further analyzed in this study show significant similarities to different plant genes. Interestingly, three different *M. truncatula* genes with similarities to plant lectins were identified. Lectins have been demonstrated to be involved in recognition processes; for example, during the legume–*Rhizobium* spp. interaction (Hirsch 1999; van Rhijn et al. 2001). Another AM-induced gene identified in this work encodes a putative nitrate transporter. Studies using ^{15}N showed that AM fungi are able to

take up ammonium as well as nitrate from soil environments (George et al. 1992; Johansen et al. 1992). The positive influence of AM on nitrogen supply to plant roots could be shown in various studies (Subramanian and Charest 1998; Tobar et al. 1994). An increased RNA accumulation of the putative nitrate transporter gene *MtGim* 7 could be detected in a fully established AM symbiosis; therefore, it could be involved in the uptake of nitrate supplied by the fungus.

Additional genes represented by *MtGim* 12, 32, and 31 have been identified as being induced during AM. The *MtGim* 12 sequence showed similarities to *syrP*, a regulatory protein of *Pseudomonas syringae* that is suggested to participate in a phosphorylation cascade controlling the production of the phytotoxin syringomycin (Zhang et al. 1997), indicating that similar regulatory pathways may occur during AM. *MtGim* 32 shares similarities with different tissue-specific proteins from two other legumes, pea and chickpea. All of these sequences share one striking feature, the presence of highly repeated sequences (Munoz et al. 1997), indicating a role as cell wall structural proteins. *MtGim* 13 (similar to the small type I copper-binding proteins, phytocyanins) and *MtGim*30 (with similarities to miraculin-like proteins and plant Kunitz-type proteinase inhibitors) also were identified as genes specifically induced during AM.

We selected a gene encoding a putative GST to localize the gene expression using promoter-reporter gene studies. GSTs are abundant proteins encoded by a highly divergent gene family (Edwards et al. 2000). Strongly increased expressions of different plant GST genes could be shown under various stress condition, such as exposure to pathogens, chemical treatments, or changes in environmental conditions (Marrs 1996; Marrs and Walbot 1997). A fragment of the potato PRP1-defense gene *gst*1 encoding a GST was induced in response to infection with different pathogens as well as AM fungi (Strittmatter et al. 1996). In contrast, the *M. truncatula* gene *MtGst*1 clearly was induced in AM but did not show RNA accumulation after pathogenic infection. Reporter gene expressions and in situ hybridization experiments showed a localization of the potato gene *gst*1 in AM tissue, but limited to certain arbuscule-containing cells (Franken et al. 2000; Strittmatter et al. 1996). In addition, the *gst*1-promoter was shown to stimulate transcription also in root apices of noninfected roots. In contrast, the *M. truncatula* gene *MtGst*1 could be shown to be transcribed in mycorrhiza root tissue, not only in arbuscule-containing cells but also in the direct vicinity of cells containing fungal hyphae. No promoter activity was detected in noninfected root areas. Therefore, it is likely that the potato gene *gst*1 and *MtGst*1 have similar GST activities, but they are not orthologous genes with identical biological functions during AM.

This study on mycorrhiza-specific transcriptional gene activation resulted in the identification of a number of genes and their strictly AM-specific expression pattern. Verification of the role of these specific gene products in mycorrhiza functioning and identification of the regulatory elements in the genome of our model *M. truncatula* surely will be the focus of future research, and this approach will give us insights into the mechanisms of AM regulation.

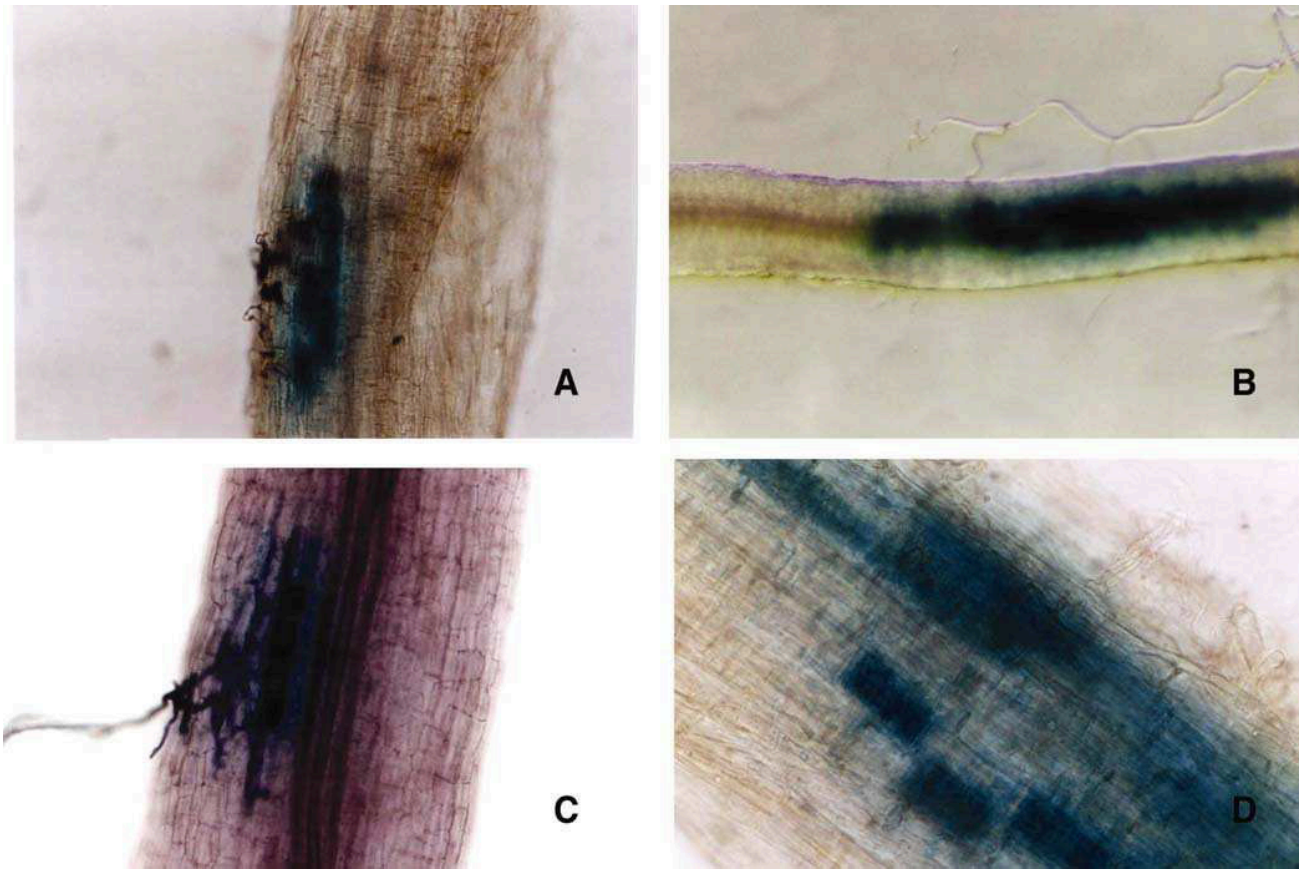

Fig. 4. *MtGst*1 promoter activity in *Glomus intraradices*–colonized hairy roots. *Medicago truncatula* plants with *Agrobacterium rhizogenes*–transformed hairy roots were colonized with *G. intraradices* and stained for GUS activity three weeks after colonization. **A,** Intense GUS activity is detectable in root parts colonized by the AM fungus. **B,** External fungal hyphae do not show GUS activity. **C,** After GUS staining, roots were subsequently stained with ink to visualize fungal structures, which appeared dark blue against the background of indigo GUS staining. **D,** Arbuscule containing cortical cells show highest promoter activity.

MATERIALS AND METHODS

Plant growth and inoculations.

Seeds of *M. truncatula* cv. Jemalong A17 were surface sterilized by 10 min of treatment with concentrated sulfuric acid, three washings with distilled water, and 5 min of incubation in 12% NaClO. Germination of the seed occurred at room temperature in the dark for 2 days.

Seedlings of *M. truncatula* were planted into a 1:2 mixture of expanded clay and vermiculite, fertilized with half-strength Hoagland's solution (Hoagland and Arnon 1950) three times a week and grown after inoculation with mycorrhiza fungi, *Sinorhizobium meliloti* or *Aphanomyces euteiches,* under constant conditions (220 μE m^{-2} s^{-1} for 16 h, 22°C, and 65% humidity). Inoculations with *G. intraradices* were carried out with a commercially available inoculum (BIORIZE Sarl, Dijon, France).

Inoculations with *S. meliloti* were carried out according to Sharypova and associates (1999). For inoculation with *A. euteiches*, 1 cm^2 of fungal mycelium grown on corn meal agar was cut out, transferred to sterile lake water, and incubated overnight at room temperature (Kjoller and Rosendahl 1998). The resulting zoospore suspension was used for inoculation of the seedlings. *M. truncatula* leaves and roots were harvested, root samples were stained with ink (Vierheilig et al. 1998), and mycorrhiza parameters were calculated following the method of Trouvelot and associates (1986).

Agrobacterium rhizogenes*-mediated transformation of *M. truncatula.

Using sequence-specific primers with restriction enzyme recognition sites at their 3′ end, 895 bp of the *MgGst*1-promoter region were cloned in front of the GUS gene in the binary vector pLP100 (Szabados et al. 1995). *A. rhizogenes* strain Arqua I (Quandt et al. 1993) was transformed with the binary plasmid using the heat shock method. *M. truncatula* roots were transformed using the protocol according to Boisson-Dernier and associates (2001).

RNA extraction.

RNA was extracted with the LiCl method (Franken and Gnädinger 1994).

SSH-cDNA library.

Total RNA (5 μg) was used to produce SMART-cDNA using the SMART cDNA synthesis kit (Clontech, Palo Alto, CA). This SMART-cDNA was used to perform an SSH using the PCR select cDNA subtractive kit (Clontech). Amplification products were cloned into the pGEM-Teasy vector (Promega, Madison, WI).

Table 2. Primer sequences used for quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

MtGim no.[a]	Primer sequences for one-step real-time RT-PCR (5′–3′)	
13	caatcttggtgcttaatacag	agcaacatacttgaatgtgag
30	tgattatcactgggacagata	catatcaacagttccacattc
11	caaaaggttcacaacttcat	cgattcctatatgagggagt
12	acaagagtgatttggagtctt	acagaatcactttctttggat
14	gagatggaattacctcttca	tgttaccgaagcaatagagtt
29	tgaaagaaacaaacatcattc	aaagactactttggtggtgat
31	cagctgaactactctttgttg	gccaagacagtatcatcaata
32	tttctatatggaggaaaaagg	tcaccattaagcaaaaagtta
3	gataaagaaactgccttcaat	aggcaaaatcaataggataag
7	agtttttctattcagaggcac	cagctgagaccttagtacctt
27 (*MtPT*4)	aatttgataggattcttttgc	ttcacatcttctcagttcttg
G. intraradices gapdh	gacgtctcagttgttgattta	tttggcatcaaaaatactaga

[a] *MtGim* = *Medicago truncatula–Glomus intraradices* mycorrhiza.

cDNA hybridizations (reverse Northern hybridization) and EST analysis.

Inserts of cDNA clones were amplified using M13 forward and reverse primer and *Taq* polymerase (Sigma, Deisenhofen, Germany). A sequence of the *M. truncatula* gene encoding the elongation factor 1 α (*MtEf-1a*) was identified using the *M. truncatula* gene index (MtGI-TC50975). A fragment of the gene was amplified from root cDNA using gene-specific primers: MtEf-1*a*for (5′–3′: caa tgt gag agg tgt ggc aat c) and MtEf-1*a*rev (5′–3′: gga gtg aag cag atg atc tgt tg). Amplification products were electrophoresed on 2% Tris-acetate EDTA gels (Sambrook et al. 1989) and transferred to nylon membranes. Digoxygenin-labeled cDNA probes were synthesized by amplification of SMART-cDNA using the PCR-digoxygenin-labeling mix (Roche Diagnostics Corporation, Mannheim, Germany). The filters were hybridized and signals were detected according to the protocol of the digoxygenin supplier (Roche Diagnostics Corporation). Sequences of cDNAs, which showed increased RNA accumulation after hybridization to the mycorrhiza cDNA probe, were used for database search to identify similarities on the amino acid level (blastx) (Altschul et al. 1997). Comparisons to the TIGR *M. truncatula* gene index (MtGI) were done in order to identify identical TC sequences in this database.

3′ and 5′ RACE.

Full-size cDNA sequences were obtained by identification of 5′ and 3′ cDNA ends using gene SMART-cDNA as a template. cDNA fragments were amplified using the SMART PCR-primer (Clontech) and gene-specific primers for amplification.

Primer selection for real-time PCR.

To optimize primer selection, sequences were aligned with the corresponding MtGI TC sequences. Criteria for primer selection were: differences in annealing temperature (T_m) must not be larger than 1°C, T_m must range between 50 and 55°C, primer lengths have to be between 17 and 25 bp, and amplification products must not be smaller than 150 bp. Results of clustering for the genes for further studies and primer selection for real-time RT-PCR are shown in Table 2. Selected primer sequences were compared to the TIGR *M. truncatula* gene index to verify primer specificity. Chosen primer sequences were not redundant in the MtGI.

RT-PCR.

Total RNA (5 μg) was treated with DNase (Bauer et al. 1993) and used for cDNA synthesis with an oligo$(dT)_{15}$ as primer in a 20-μl volume following the protocol of the supplier of the MMLV-reverse transcriptase (Promega). This cDNA was diluted 1:10 in water and 1 μl was used directly for PCR in a 20-μl volume with 1 U *Taq* polymerase (Sigma, Deisenhofen, Germany), 200 μM dNTPs, and 1 μM of the respective primers, MtGst1for (5′–3′: gga gac aat gtg gtt gtt ttg g) and MtGst1rev (5′–3′: acca gaa atc cca gag gtg att c). Amplifications were carried for 5 min at 95°C followed by 30 cycles (30 s at 94°C, 30 s at the specific annealing temperature, and 30 s at 72°C) and a final extension for 5 min at 72°C. Amplification products were directly analyzed by 2% agarose gel electrophoresis.

Quantitative real-time RT-PCR.

Total RNA (100 ng) was added to each amplification reaction. Quantitative real-time RT-PCR was carried out using the Opticon real-time cycler (MJ Research, Waltham, MA, U.S.A.) and QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). The following run protocol was used: reverse transcription (50°C for 30 min), initial denaturation (95°C for 15

min), amplification and quantification program repeated 44 times (94°C for 15 s, 45°C for 30 s, 72°C for 30 s, with a single fluorescence measurement), and melting curve program (40 to 95°C, with fluorescence read every 1°C).

Inverse PCR.

Genomic *M. truncatula* DNA (2 µg) was cut with the restriction enzymes *Dra*I, *Eco*RV, *Nde*I, *Nhe*I, or *Nsi*I. Circularization of 50 ng of digested DNA was carried out overnight in 30 µl using 0.6 U T4 DNA ligase at 15°C. The flanking regions were amplified with the Expand High Fidelity System (Roche Diagnostics) using 10 ng of recircularized DNA for each reaction and outward orientated primers.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

TIGR *M. truncatula* gene index: www.tigr.org.

Chapter 3

A member of the germin-like protein family is a highly conserved mycorrhiza-specific induced gene.*

Doll J, Hause B, Demchenko, K, Pawlowski K, Krajinski F

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Chapter 4

Insights into the transcriptional regulation of *MtGst*1 - an AM-specific gene of *Medicago truncatula*.

Doll J, Wolff S, Tiller N, Krajinski F

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Chapter 4

Insights into the transcriptional regulation of *MtGst*1

- an AM-specific gene of *Medicago truncatula*

author for correspondence:

PD Dr. Franziska Krajinski

Lehrgebiet Molekulargenetik

Universität Hannover

Herrenhäuser Str. 2

D-30149 Hannover

Germany

E-mail: krajinski@lgm.uni-hannover.de

Phone: +49-511-762 5548

Fax: +49-511-762 4088

Subject areas:

environmental and stress responses, gene regulation

Authors:

Jasmin Doll, Stephanie Wolff, Nadine Tiller and Franziska Krajinski*

Lehrgebiet Molekulargenetik, Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany

*corresponding author

Chapter 4

Abstract

In this study, the AM-specific gene *MtGst*1 of *Medicago truncatula* will be further characterized with respect to its specificity and regulatory mechanism. Using reporter studies in transgenic hairy roots of *Nicotiana tabacum,* heterologous promoter activity occurred in mycorrhizal root cells indicating that the observed transcriptional induction is not legume specific. The absence of promoter activity in *M. truncatula* root nodules underlined the specificity of AM-induction with respect to other symbiotic associations. Furthermore, treatments with different phytohormones did not lead to *MtGst*1 transcription; this also strengthened the AM-specificity of the *MtGst*1 promoter. In order to narrow down the promoter area providing this AM-specific transcription, promoter deletion studies were carried out. The promoter area necessary for the transcriptional induction could be restricted to 57 bp located at -413/-356 bp of the promoter. Electrophoretic mobility shift assays (EMSA) were used to localize putative AM-response elements in the promoter of *MtGst*1 to which putative mycorrhiza-specific transcription factors could bind. This led to potential response elements in the position -433 to -198 bp according to the transcription start suggesting the binding sites for putative positive-regulating mycorrhiza-specific transcription factors in this area.

Keywords:

arbuscular mycorrhiza, EMSA, glutathione-S-transferase, *Medicago truncatula*, promoter

Abbreviations:

AM: arbuscular mycorrhiza, EMSA: electrophoretic mobility shift assay

Chapter 4

Introduction

The arbuscular mycorrhiza (AM) is a mutualistic symbiosis with its origin in the Devonian period, corresponding with the appearance of terrestrial plants. This could be proven by fossil records showing AM-fungi associated together with the root systems of first land plants several million years ago (Remy *et al.*, 1994).

Nowadays the formation of an AM with these obligatory biotrophic fungi of the order *Glomeromycota* is widespread among terrestrial plants (Schüssler *et al.*, 2001). More than 80% of all vascular plants are capable of forming an interaction of this kind (Newman and Reddell, 1987). Thus, it can be assumed that key mechanisms of molecular regulation during the interaction are conserved in different host plant species.

Colonization of the plant roots with AM-fungi can greatly improve plant growth and yield. The mechanisms by which the plant and symbiont benefit from the association are complex (Merryweather and Fitter, 1995; Smith and Gianinazzi-Pearson, 1988). However, it was shown that the symbiotic fungal partner can mediate higher levels of plant resistance against stress conditions (Augé, 2001). The plants also seem to be better protected against pathogenic attack while having an interaction with AM-fungi (Slezack *et al.*, 2000). The major characteristic of this interaction is the bi-directional nutrient exchange in which both partners benefit. In this endosymbiosis an extensive external fungal mycelium is associated with the colonized host root and the fungal partner of this symbiosis provides nutrients to its host and in return carbohydrates are supplied to the micro-symbiont (Smith and Read, 1997). Inorganic phosphate, but also increased uptake of other minerals by the fungi, plays the most important part in this nutrient transfer from fungi to plant (Karandashov, 2004).

Currently, the physiology of the AM-symbiosis is well characterized but the understanding of the underlying molecular mechanisms is just in its infancy. The identification of AM-specifically

expressed genes has provided a strong starting point and further characterization of these genes will lead to a better understanding of the regulation of the symbiosis. To date, the results of diverse experimental and *in silico* analyses had identified several specifically regulated genes in the AM-symbiosis which are not induced after pathogen infection or during other symbiotic interactions (Frenzel *et al.*, 2005; Salzer *et al.*, 1999; Wulf *et al.*, 2003).

The nutrient exchange is the major characteristic of the AM-interaction. Several research groups were concentrating on the identification of corresponding mycorrhiza-specific transporters, particularly phosphate transporters. Up to now, several mycorrhiza-specifically induced genes were identified. Currently, knowledge of the regulatory mechanisms that control mycorrhiza-specific expression is limited. Only a confined number of mycorrhiza-specific promoters were analyzed and revealed AM-specific expression patterns (Harrison *et al.*, 2002; Nagy *et al.*, 2005; Rausch *et al.*, 2001; Vieweg *et al.*, 2004; Wulf *et al.*, 2003).

In earlier studies a mycorrhiza-specifically induced gene for a glutathione-S-transferase has been identified suggesting an important role for this interaction. It was demonstrated that the promoter of *MtGst*1 was sufficient for exclusive *MtGst*1-expression in mycorrhizal tissue (Wulf *et al.*, 2003).

In order to assess the involvement of *MtGst*1 in an AM-symbiosis, we embarked on a study of the promoter to identify *cis*-acting control elements that regulate AM-specific expression. Using deletion analyses of p*MtGst*1 fragments, we describe specific response elements within the promoter that govern the regulation of *MtGst*1 and control AM-specific expression. Electrophoretic mobility shift assays were performed to identify putative transcription factor binding sites in the promoter sequence of *MtGst*1.

*MtGst*1 was expressed in all zones of the root cortex colonized by *Glomus intraradices*, but not during pathogenic interactions as was shown for the potato GST gene PRP in addition to AM-expression (Strittmatter *et al.*, 1996). No *MtGst*1 expression was detectable by RNA

accumulation studies during the symbiotic interaction with Rhizobia or by increased phosphate nutrition (Wulf *et al.*, 2003). Since plant hormones seem to be involved in the development and regulation of an AM-symbiosis, we analyzed the transcription of *MtGst*1 relating to different phytohormone treatments. On the cellular level, we provide evidence that *MtGst*1 is highly expressed in arbuscule-containing cells of mycorrhizal roots, both in the model legume *Medicago truncatula* and in *Nicotiana tabacum* as a non-legume plant. However, promoter activation was not found in nodules formed in the interaction between *M. truncatula* roots and *Sinorhizobium meliloti*. The observed expression regulation implies that there is an underlying conserved mechanism.

Materials and Methods

Plant growth and inoculations

Medicago truncatula cv. Jemalong A17 seeds were sterilized for 10 min in sulphuric acid, washed in water and finally treated with 6% NaOCl (v/v) for an additional 5 min. Seeds were germinated first in the dark for three days and then in light for two days.

Plants were fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1950) twice a week altogether three weeks long. For the phytohormone treatment of the plants, different phytohormones (50 µM 2,4D, 50 µM kinetin, 50 µM GA_3, 75 µM ABA, 50 µM jasmonate) were added to the fertilizer.

Seeds of *Nicotiana tabacum* var. Samsun were sterilized for 1 min in 70% ethanol and washed three times with water, then treated with 6% NaOCl (v/v) for 10 min.

The seeds germinated on M&S media at 23°C, 8 h light and 8 h dark. Plants were inoculated as described earlier by Doll *et al.* (2003) with the mycorrhiza fungus *Glomus intraradices* using a commercially available inoculum by Biorize Sarl (Dijon, France). Plants were harvested after 21 days after inoculation. Selected root segments were ink-stained according to Vierheilig *et al.* (1998) to calculate the mycorrhizal colonization intensity (Trouvelot *et al.* 1986).

RNA extraction and cDNA synthesis

Plants were harvested 21 days after inoculation and phytohormone treatment respectively. RNA was extracted using a LiCl precipitation method (Franken and Gnädinger, 1994). According to the supplier of the MMLV-reverse transcriptase (Promega, Mannheim, Germany) cDNA was synthesized with five µg of total RNA with an oligo(dT)15 as primer in 25 µl volume.

RT-PCR

One µl of a 1:10 dilution of the resulting cDNA was used for PCR in 20 µl volume using 1 U

Taq polymerase, 200 µM dNTPs and 1 µM of the respected primer pairs. The constitutively expressed translation elongation factor 1 alpha (*MtEf*1) was used as positive control, whereas the mycorrhiza-specific phosphate transporter (*MtPT*4) was used as control for the mycorrhization. *MtGst*1-specific primers were used to amplify *MtGst*1-transcripts. Following primer pairs were used: 5´to 3´ *MtEf*1 for CAA TGT GAG AGG TGT GGC AAT C and *MtEf*1 rev GGA GTG AAG CAG ATG ATC TGT TG; *MtPT*4 for GTC GCC TTG TTT GGA ACA TTC CCC GG and *MtPT*4 rev TCA CAT CTT TC AGT TCT TGA GTC C; *MtGst*1 for TGA AAG AAA CAA ACA TCA TTC and *MtGst*1 rev AAA GAC TAC TTT GGT GGT GAT.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were done using the DIG Gel Shift Kit, 2nd Generation by Roche (Penzberg, Germany). The promoter fragments were amplified by PCR using primers for sequences of around 150 bp overlapping each other for about 50 bp using specific primers (forward / reverse primers, 5' to 3': CCT AGT AAA CAT CGT AGT C / GGA CTA TCT TTG TTG CTT GTG for fragment 1 closest to the start codon, TCC TCT CAT CCC ACG TCC AA / AAT TGT GTG AGA GTT GTT G for fragment 2, TCC TAG TTT GGA TAC ATC GC / GTA CAA TGT TGT AAG TGT CC for fragment 3, GAG AAG CTC TTT GAT GTG TG / GGC CGG CCT TAA GCT AC for fragment 4, GAA GTA AGG AAC CAT GTC / GAT ACA TTT GAC AAC ACA TC for fragment 5, GTA TGT GTC CGA AAA ATG T / GTA GTC TAA TGG AAC TTG for fragment 6, CAT AGA GATTTG GAC AC / GTC TTG AAG ATC CGC G for fragment 7, GAA GCC ATG ACG GAA GCT TGG G / GAA ATA AAG TGT GCT TTT TTT GTG for fragment 8, GCT TTA AGA GGA TGT TTT G / CTC CTCAAT TGA AGG CT for fragment 9, AGG CCC TTA ATT TTC TTG TTA GA / CAA TGT GTC ACT CAG GC for fragment 10). Binding reactions were carried out with 0.8 ng DIG labeled oligonucleotides and 5 µg crude protein extract from mycorrhizal and non-mycorrhizal roots or without any protein. Binding reaction

was carried out for 20 min at room temperature. Samples were applied to an 8% (v/v) non-denaturing polyacrylamide gel. DNA was electro-blotted on a nylon membrane and signals were detected on X-ray film according to the manufacturer of the DIG-system (Roche Diagnostics, Mannheim, Germany).

Cloning of the *MtGst*1 promoter

Amplification of the *MtGst*1 promoter was done as previously described using specific primers deduced from the sequence obtained via inverse PCR technique (Wulf *et al.,* 2003). For the p*MtGst*1 deletion constructs promoter sequences were amplified using specific primers (5' to 3': AGG CCC TTA ATT TTC TTG for 868 bp of the promoter p*MtGst*1(-836/+32), TTG AGG AGT AAT TTT TGT TTG ACA TAG A for 674 bp of the promoter p*MtGst*1(-640/+35), TGT ATG TGT CCG AAA AAT GTA TCC for 558 bp of the promoter p*MtGst*1(-523/+35), CAT TCT GAA GTA AGG AAC CAT GTC CAC for 481 bp of the promoter p*MtGst*1(-446/+35), GTA TAC AAG TTC CAT TAG ACT ACT TAA for 448 bp of the promoter p*MtGst*1(-413/+35), GAG AAG CTC TTT GAT GTG TTG for 388 bp of the promoter p*MtGst*1(-356/+32), TCC TAG TTT GGA TAC ATC GCC for 287 bp of the promoter p*MtGst*1(-255/+32), TCC TCT CAT CCC ACG TCC AA for 208 bp of the promoter p*MtGst*1(-176/+32) and for reverse primers ATG ATT CTT TGG ACT ATC for p*MtGst*1(+15/+32) and GCA ATG ATT CTT TGG ACT ATC for p*MtGst*1(+15/+35)). PCR products of 868, 388, 287, 208 bp had additionally restriction sites for *EcoR*I and *BamH*I at the 5´end of the primers. PCR-products were cloned into pGEM-T-easy (Promega, Mannheim, Germany) and the vectors were restricted with *EcoR*I and *BamH*I. The restricted promoter sequences were cloned in front of the GUS gene into the corresponding sites of the pLP100 vector (Szabados *et al.*, 1995). The 674, 558, 481 and 448 bp PCR fragments were cloned into the binary vector pMDC163 (Curtis and Grossniklaus, 2003) using the GATEWAY cloning technique (Invitrogen, Karlsruhe, Germany). The primer

for these promoter fragments were modified with attachment B sites according to the manufacturer. *Agrobacterium rhizogenes* strain ARqua1 (Quandt *et al.*, 1993) was transformed with the binary vectors via electroporation.

Agrobacterium rhizogenes*-mediated transformation of *M. truncatula

According to a modified protocol of Vieweg *et al.* (2004), we transformed *M. truncatula* roots of 5-day-old A17 seedlings with the *Agrobacterium rhizogenes* strain ARqua1 containing the binary vectors. The roots of two to three week old *N. tabacum* seedlings were cut 0.5 cm above the root tip and dipped into the appropriate *Agrobacterium* solution. Transformed seedlings were incubated for 3 weeks at 24°C and a 16h/8h light/dark period vertically on Farhaeus medium to induce hairy roots. After cutting the main root of promoter-GUS-containing transformants to promote growth of lateral roots, the plants were mycorrhized for 21 days.

Histochemical staining of hairy roots

A GUS-staining was performed to investigate activation of the *uidA* gene. Histochemical assays for the detection of the GUS-activity were performed as described by Jefferson *et al.* (1987). Roots were incubated staining solution at 55°C for 1h than for another 1 to 2 h at 37°C. Subsequently, roots were cleared and stored in 70% ethanol. In order to verify the presence of the fungus, GUS-stained roots were stained with Acid-Fuchsin (Gerdeman, 1955).

Computational sequence analyses

Standard computational DNA modifications were performed using the Vector NTI software (Invitrogen). Promoter sequences were analyzed using MatInspector to identify common promoter motifs (Quandt *et al.*, 1995).

Results

*MtGst*1 is AM-specific but not legume-specific

Recently, a glutathione-S-transferase gene of *Medicago truncatula* has been identified to be transcribed mycorrhiza-specifically with respect to phosphate nutrition and other plant-microbe associations (Wulf *et al.*, 2003). To strengthen the AM-specificity of *MtGst*1, we studied the transcriptional regulation of *MtGst*1 under different phytohormone treatment of the plants compared with plants inoculated with the AM-fungus *Glomus intraradices*. Mycorrhizal parameters after three weeks were calculated according to Trouvelot *et al.* (1986): F=100%; M=49.61%; m=49.61%; A=24.05%; a=48.48%. RNA-accumulation studies revealed that the treatments with 50 µM 2,4D; 50 µM kinetin; 50 µM $GA_{3;}$ 75 µM ABA or 50 µM jasmonate did not induce an *MtGst*1 transcription. In contrast, *MtGst*1 transcripts were detectable in mycorrhizal roots by RT-PCR (Fig. 1).

The promoter of the AM-specific gene *MtGst*1 provided strong activation in mycorrhizal root tissue, but mainly in arbuscule-containing cells (Wulf *et al.*, 2003). To find out whether the regulatory mechanisms leading to the observed AM-specific transcriptional induction are conserved between different plant species, the promoter-GUS construct was used to compare the activation of p*MtGst*1 in *Nicotiana tabacum* as a non-legume with the activation in *M. truncatula*. Plants were transformed with *Agrobacterium rhizogenes* producing transgenic roots containing p*MtGst*1 (-836/+32) fused in front of the GUS reporter gene. Identical reporter gene activation patterns were observed in mycorrhizal root tissue of *M. truncatula* and *N. tabacum* (Fig. 2A).

To find out whether the promoter activation is mycorrhiza-specific with respect to other symbiotic associations, we examined the promoter activity in *M. truncatula* roots in response to the infection with *Sinorhizobium meliloti*. No GUS-activity was detectable in transgenic nodules

(data not shown) suggesting that the observed transcriptional activation is a mycorrhiza-specific mechanism conserved also in non-legume plants.

Analysis of the *MtGst*1 promoter

Recently, first promoter analyses of *MtGst*1 were presented where root fragments, which harbored arbuscules, showed strong GUS-activities using almost 900 bp of the promoter sequence in front of the GUS-reporter gene (Wulf *et al.*, 2003). In order to identify the *MtGst*1 promoter region responsible for this activation pattern in mycorrhizal roots, promoter deletion fragments were constructed varying in length. 868, 674, 558, 481, 448, 388, 287 and 208 bp of the *MtGst*1 promoter sequence were cloned in front of a *uidA* reporter gene (Fig. 2C). *M. truncatula* roots were transformed with these deletion constructs and 3 weeks after inoculation with *Glomus intraradices,* the transgenic hairy roots were stained for GUS-activity. The 448 bp of p*MtGst*1 were enough to activate the *uidA* gene giving a strong GUS-expression in arbuscule-containing cells (Fig. 2B). Using shorter deletion fragments of 388 bp, 287 bp and 208 bp size, no GUS-activity could be observed in any tissue (data not shown). The promoter activity of the 448 bp deletion construct was identical with respect to strength and pattern as the observed activity of the larger fragments. This means that the deletion did not have any effect of the strengths of p*MtGst*1. Hence, the promoter area necessary for the AM-specific transcriptional activation of p*MtGst*1 is located in the -413 to -356 area in relation to the start codon. Within this 57 bp region potential *cis*-acting AM-responsive control elements seem to be most likely.

Putative protein-binding promoter sequences

The presented promoter deletion analyses suggested the presence of AM-responsive elements, putative positive regulator binding sites, within a limited promoter area. A determination of these putative protein-binding sites was carried out by Electromobility Shift Assay (EMSA).

Therefore, the p*MtGst*1 was parted in 150 bp long fragments overlapping on both sides with 50 bp (Fig. 3A). The fragments were labeled and incubated with crude proteins extracted from either mycorrhizal or non-mycorrhizal *M. truncatula* roots. A control reaction was carried out with labeled promoter fragments without any protein extracts to exclude artificial shifts of the gel composition.

Strong specific gel shifts could be observed for fragments 4, 5 and 6 covering the promoter region -198 to -521 bp after incubation with proteins from roots inoculated for 3 weeks with *Glomus intraradices* (Fig. 3B). No signals were detectable after incubation with proteins of non-mycorrhizal roots or without protein extracts, supporting the specificity of the observed gel-shift after incubation with the AM-proteins.

Further promoter analysis

A schematic representation of the results of deletion and gel-shift analyses is shown in figure 4A. The observed results indicate that at least two regulators bind to the -413/-198 area to induce the transcription. Computational analyses were done in order to identify conserved motifs within the promoter sequence. First of all, a consensus sequence of a TATA-box (TATAAAT) in the area of -35/-29 bp according to the transcription start could be identified. MatInspector analysis of the -413/-198 area revealed a nodulin consensus sequence and a plant-specific zinc-finger-type factor (WRKY) associated with pathogen defense (Fig. 4B). Additionally, an organ-specific element (OSE) was identified “CTCTT”. OSE are characteristic elements of leghemoglobin and other nodulin gene promoters (Stougaard *et al*., 1987).

Recently, different mycorrhiza-specific promoter motives were described (Boisson-Dernier *et al.*, 2005; Fehlberg *et al.*, 2005), but none of these sequences were detectable in the promoter of p*MtGst*1.

Discussion

The arbuscular mycorrhiza is an ancient symbiosis originating in the Devonian era more than 400 million years ago. All those years of co-evolution of plants and AM-fungi suggests that conserved mechanisms of the AM-development and specific regulation exist in closely related as well as in evolutionary distant plant species. Recently, investigations on AM-induced genes within different plant species support this hypothesis. A comparison of the regulation of different mycorrhiza-specific phosphate transporter genes in phylogenetically distant plant species confirmed a conserved mechanism (Karandashov *et al*., 2004). Similar results were reported by Vieweg *et al.* (2004) for the promoter of the leghemoglobin gene, *VfLb*29, induced in both, roots of *Medicago truncatula* and *Nicotiana tabacum*.

Recently, an AM-specifically induced glutathione-S-transferase gene in *M. truncatula*, *MtGst*1 was identified indicating a strong activation in mycorrhizal root tissue (Wulf *et al.*, 2003). Glutathione-S-transferases (GSTs) belong to a large gene family in plants. Several members have been shown to be stress-regulated genes for a wide variety of stimuli, such as plant hormones, pathogen and abiotic stresses. Various family members show diverse expression patterns (Edwards *et al.*, 2000; Marrs, 1996; Seppanen *et al.*, 2000). For a GST in potato, it was demonstrated that this *gst*1 gene is regulated after infection with different pathogens as well as in the mycorrhiza interaction (Strittmatter *et al.*, 1996). In contrast, the *M. truncatula* gene, *MtGst*1, was exclusively induced in mycorrhizal roots and not after pathogenic attack (Wulf *et al.*, 2003). In this report, we extended the characterization and specificity of the *MtGst*1 regulation.

The mycorrhiza-specific activation of the *MtGst*1 promoter led to the question whether the observed mycorrhiza-specific gene regulation is conserved in non-leguminous plants. Therefore, we transformed *N. tabacum* with the heterologous promoter reporter construct containing p*MtGst*1 (-836/+32). Identical expression patterns were observed in transgenic roots of both

plant species. Thus, a conservation of the mechanisms leading to an induction of *MtGst*1 transcription in mycorrhizal roots is likely.

Many similarities exist between both the AM-interaction and the Rhizobia symbiosis. Several genes have been identified to be induced in both interactions (Brechenmacher *et al.*, 2004; Journet *et al.*, 2001; Sanchez *et al.*, 2004; van Rhijn *et al.*, 1997; Vieweg *et al.*, 2004). Genetic evidence suggests that common signaling pathways exist (Kistner and Parniske, 2002). No reporter gene activation was detectable in nodules after inoculation with symbiotic Rhizobia, further underlining the AM-specificity of the *MtGst*1 promoter with respect to other symbiotic root-microbe associations.

Since an induction of a gene in mycorrhizal roots can also be the consequence of changed phytohormone levels within AM-roots, the influence of different hormones on the transcription of *MtGst*1 was analyzed. In contrast to mycorrhizal roots, no *MtGst*1 transcripts were detectable in *M. truncatula* roots after treatment with different phytohormones (Fig. 1) indicating that the analyzed plant hormones do not directly influence the activity of the *MtGst*1 promoter.

To unravel the molecular mechanism of the transcriptional regulation in the mycorrhiza symbiosis, the promoter of *MtGst*1 was analyzed in detail to get a better understanding of the regulatory mechanisms. The promoter deletion studies of this report suggest that promoter areas, essential for an AM-specific expression of the *MtGst*1 promoter, are located in the sequence between the two constructs -413/+35 showing reporter gene activation and -356/+32 with no transcription of the reporter gene. Potential *cis*-acting control elements seem here to be most likely.

In order to identify AM-specific response elements representing protein-binding sites for putative mycorrhiza-specific transcription factors, we arranged an Electrophoretic Mobility Shift Assay (EMSA). Binding of proteins to the promoter was observed at 3 overlapping fragments. Since there was only a signal after incubation with proteins deriving from crude extract of

mycorrhizal roots, it can be assumed that these fragments contain putative binding sites for AM-specific transcription factors.

Moreover, we continued with computational sequence analyses of the promoter of *MtGst*1 to identify conserved regulatory elements responsible for the mycorrhiza-specific expression. Computational analyses were done in order to identify conserved motifs within the promoter sequence. Analysis of the -413/-198 area revealed 2 common motifs. One of these motifs represents the core sequence of WRKY, a plant-specific zinc-finger-type factor associated with pathogen defense. This common motif is also present in the promoter sequence of *MtENOD11* (Boisson-Dernier *et al.*, 2005). For *MtENOD11*, it was shown that its promoter contains regulatory sequences necessary for gene expression during an AM-interaction. In parallel, we searched the promoter for consensus sequence motifs already known in other promoters of AM-induced genes. Organ-specific elements (OSE) with two consensus sequences "AAAGAT" and "CTCTT" (Stougaard *et al*., 1987) are characteristic elements of leghemoglobin and other nodulin gene promoters. In the -413/-198 area, we could identify one OSE consensus sequence "CTCTT". Since we did not observe any activity of p*MtGst*1 in nodules, the role of this motive has to be verified in future.

Recently, mycorrhiza-specific AT-rich consensus sequences were described (Boisson-Dernier *et al.*, 2005; Fehlberg *et al.*, 2005). Computational analysis of the promoter sequence -413/-198 did not reveal previously characterized mycorrhiza-specific consensus sequences in the promoter of p*MtGst*1.

In this report, we described the identification of a short promoter area, which is responsible for the AM-specific *MtGst*1 regulation. This led to potential response elements suggesting the binding sites for putative positive-regulating mycorrhiza-specific transcription factors.

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Figure Legends

Figure 1: *MtGst*1-RNA accumulation in *Medicago truncatula* roots after treatment with different phytohormones

Transcripts of *MtGst*1, *MtPT*4 and *MtEf*1 were amplified by RT-PCR with RNA of uninfected plant roots (1), mycorrhizal roots (2) and different treated plants with phytohormones, like 50 µM kinetin (3), 50 µM GA_3 (4), 75 µM ABA (5), 50 µM 2,4D (6),50 µM jasmonate (7). M, 100 bp Plus marker. Amplification with *MtGst*1 and *MtPT*4 specific primers revealed a strong band exclusively in mycorrhizal roots and not in untreated roots or roots with different phytohormone treatment.

Figure 2: Promoter-reporter analyses in transgenic hairy roots of *Medicago truncatula* and *Nicotiana tabacum*

Roots were transformed and 3 weeks after inoculation with *Glomus intraradices*. GUS-staining was performed.

A: Mycorrhizal transgenic roots of *Nicotiana tabacum* carrying the heterologous p*MtGst*1 (-836 /+32) promoter fused to the GUS reporter gene. Promoter activity could be observed in a similar pattern than in *M. truncatula* hairy roots.

B: GUS-activity of transgenic mycorrhizal hairy roots of *M. truncatula* transformed with the construct of p*MtGst*1 (-413/+35). *MtGst*1 promoter activity is detectable in cells colonized by the AM-fungus. Strong signals appear in arbuscule-containing cells.

C: Different promoter deletion constructs fused to a *uidA* reporter gene. Promoter lengths are given according to the transcription start. The promoter activity in mycorrhizal roots is indicated for each construct. The region -413/-356 necessary for AM-specific activation is encircled.

Figure 3: Electrophoretic mobility shift assay (EMSA) of p*MtGst*1

A: The promoter sequence of *MtGst*1 was parted into 150 bp long fragments with 50 bp overlaps on each side. Positions relative to the transcription start: **1** (-84/+35); **2** (-176/-8); **3** (-257/-121); **4** (-356/-198); **5** (-439/-325); **6** (-521/-391); **7** (-617/-465); **8** (-681/-551); **9** (-726/-633); **10** (-836 /-710).

B: For the binding reaction 0.8 ng of DIG-labeled promoter fragments were accomplished with protein extract from mycorrhizal (M) or non-mycorrhizal (C) *M. truncatula* roots and without proteins as control (0). Fragments 4 to 6 (in total -191 to -523 bp upstream of the transcription start) give a specific signal after incubation with protein extract from mycorrhizal roots and no signals were detectable after incubation with proteins of non-mycorrhizal roots or in the control experiment without protein extract.

Figure 4: Sequence of the *MtGst*1 promoter

Indicated positions are relative to the transcription start (+1).

A: Schematic representation of the promoter sequence. Promoter area necessary for AM-specific transcriptional induction as revealed by deletion analyses is shown in black (-413/-356). Promoter area not sufficient for reporter gene activation is shown in grey. EMSA fragments giving a specific signal after incubation with protein extract from mycorrhizal roots are indicated (-521/-391; -439/-325; -356/-198).

B: Promoter sequence of *MtGst*1. 5´untranslated region (5´ UTR) is in italics and the start codon is in bold. The region -413/-356 (57 bp) necessary for AM-specific activation according to the promoter deletion analysis is marked with a dotted line. Additionally, the area -413/-198 evoked by deletion and EMSA analysis is marked with bold letters. The consensus sequence for the TATA-box and the core sequences for the potential common motives WRKY, OSE and NCS are highlighted.

Figure 1

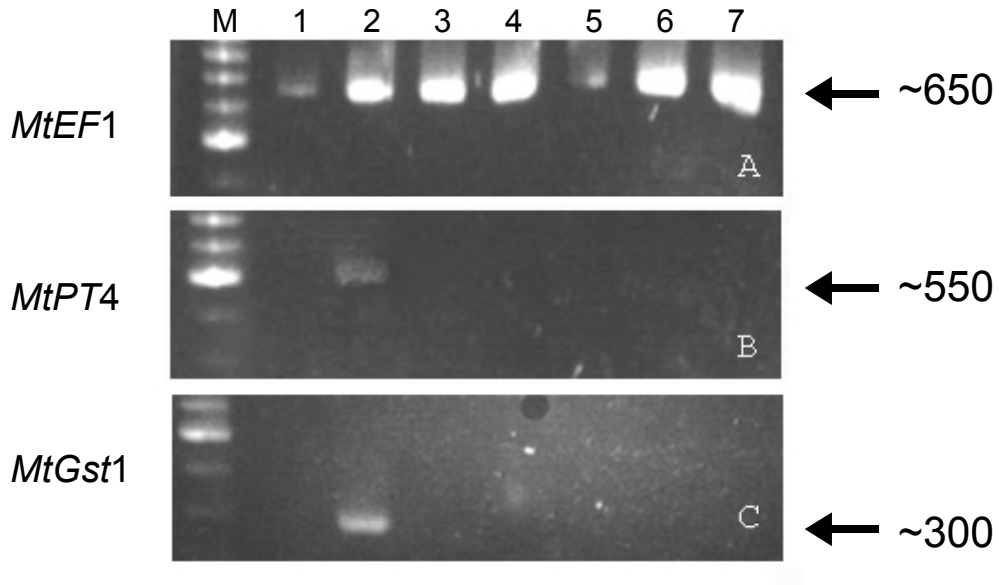


Figure 2

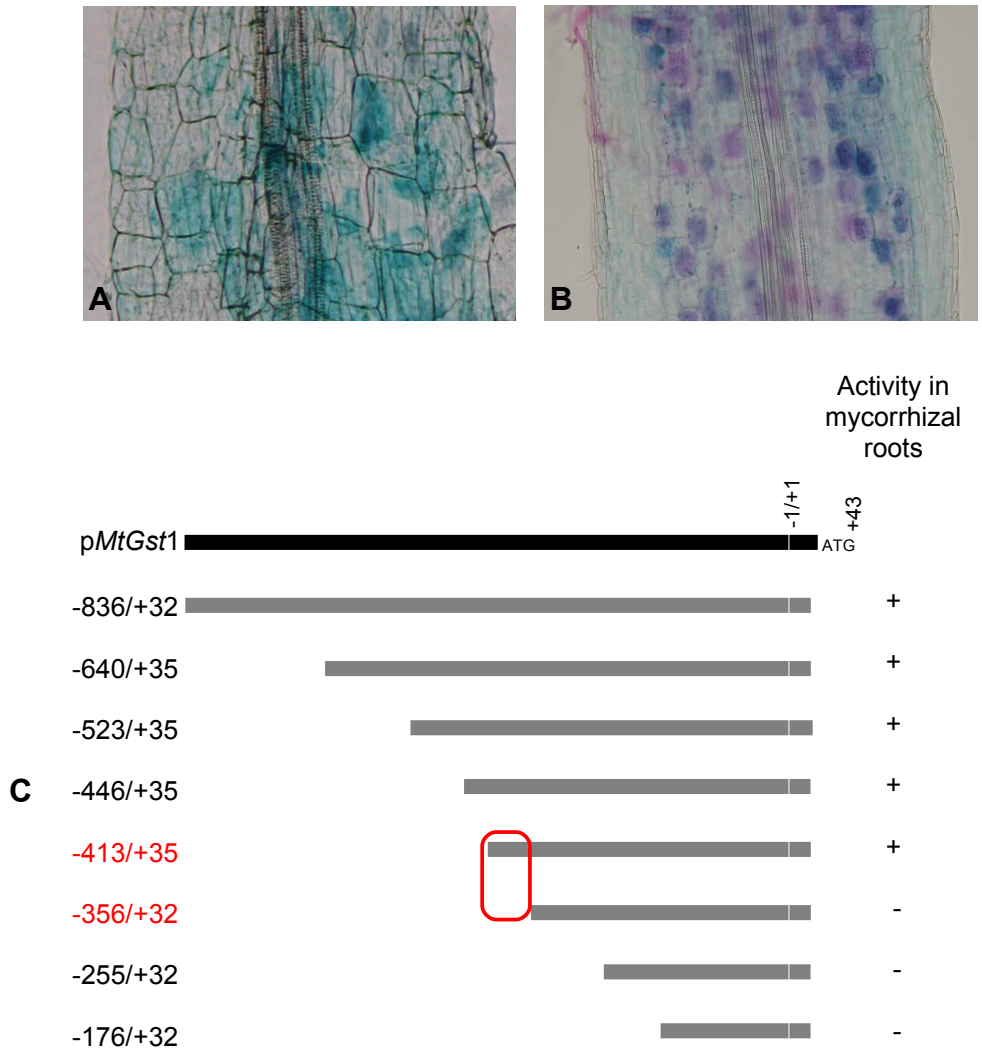
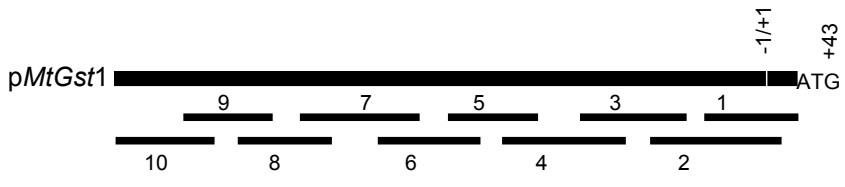

A
B
Activity in mycorrhizal roots
-1/+1
+43
pMtGst1
ATG
-836/+32
-640/+35
-523/+35
C
-446/+35
-413/+35
-356/+32
-255/+32
-176/+32
+
+
+
+
+
-
-
-

Figure 3

A



B

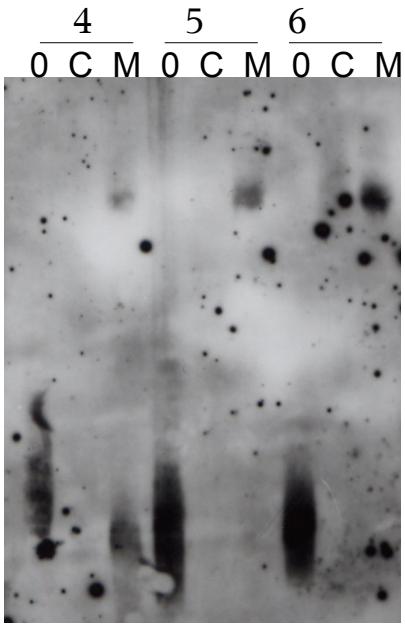
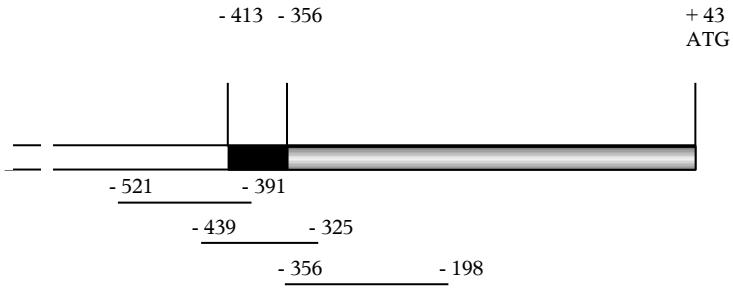


Figure 4

A



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-586   CACACAAAAA AAGCACACTT TATTTCTAAA AAATAAATAT CATTTAATTA
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-486   ATTTTTCGCG GATCTTCAAG ACAAAATATT GTCATAATAT CATTCTGAAG
-436   TAAGGAACCA TGTCCACATA AAAGTATACA AGTTCCATTA GACTACTTAA
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- 86   CCTAGTAAAC ATCGTAGTCT AATTTTTTTA TTTATCTAAA CTAAACTTTC
- 36   CTATAAATTT CAACAACTCT CACACAATTT CAAGTCATCA CAAGCAACAA
+ 15   AGATAGTCCA AAGAATCATT GCTAACATG
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Chapter 5

PTGS approach to unravel the function of *MtGlp*1 and *MtGst*1, two AM-specific *Medicago truncatula* genes.

Doll J, Xu Y, Krajinski F

in preparation

Chapter 5

in preparation:

PTGS approach to unravel the function of *MtGlp*1 and *MtGst*1, two AM-specific *Medicago truncatula* genes (working title)

Author for correspondence:
PD Dr. Franziska Krajinski
Lehrgebiet Molekulargenetik
Universität Hannover
Herrenhäuser Str. 2
D-30149 Hannover
Germany
E-mail: krajinski@lgm.uni-hannover.de
Phone: +49-511-762 5548
Fax: +49-511-762 4088

Subject areas:
environmental and stress responses, reverse genetics, functional analysis

Abbreviations:
AM: arbuscular mycorrhiza, PTGS: Post-transcriptional gene silencing, RNAi: RNA interference

Authors:
Jasmin Doll, Yan Xu and Franziska Krajinski*

Lehrgebiet Molekulargenetik, Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany

*corresponding author

Abstract

Several reverse genetic techniques have been used to study the function of genes of interest. Recent studies have shown that the formation of double stranded RNA (dsRNA) can initiate mRNA degradation of the corresponding target sequence. This gene silencing mechanism taking place at the post-transcriptional level occurs in many organisms. Nowadays, the PTGS phenomenon is widely used as a tool to reduce or silence the expression of a target gene.

As yet, there is sparsely known about the function of genes specifically regulated in an AM-symbiosis. To understand the role of AM-specific genes, we have chosen a PTGS approach in whole transgenic plants. We have selected two candidate genes already characterized in earlier projects: *MtGlp*1, a gene with high similarity to germin-like proteins, and a gluthatione-S-transferase gene of *M. truncatula, MtGst*1. Both genes are specifically expressed in arbuscular mycorrhiza symbiosis. However, so far the functional role of these genes has not been determined.

Several transgenic *M. truncatula* plants representing putative PTGS lines were established. First analyses of these plants could prove the integration of the T-DNA containing sense and antisense sequence of *MtGlp*1 and *MtGst*1 respectively flanking an intron. Different approaches, especially with a quantitative real-time PCR, indicated different reduced transcript levels of *MtGlp*1 and *MtGst*1. These results indicate that PTGS is a powerful tool to knock down genes in *M. truncatula.*

Keywords: Arbuscular Mycorrhiza, Germin-like protein, Glutathione-S-transferase, *Medicago truncatula*, Post-transcriptional gene silencing, RNA interference

Introduction

Arbuscular mycorrhiza (AM) fungi play an important role in the life of plants. Plants forming an AM possess an increasing tolerance and resistance to biotic stress conditions (Augé, 2001), especially roots against pathogenic attack (Slezack *et al.*, 2000). Moreover, plants living in symbiosis with AM-fungi can greatly improve in growth and yield. One reason for this is the bi-directional nutrient exchange taken place in this mutualistic interaction. The obligate biotrophic fungi are not able to obtain carbon essential for its life cycle without symbiotic plant partners (Smith *et al.*, 2003). AM-fungi facilitate mineral nutrients which they transfer from the soil to the plant roots (Pfeffer *et al.*, 1999). Improvements in phosphorus acquisition are significantly important for plant growth and health (Smith and Read, 1997). In many soils, the concentration and the availability of phosphorus to plants is the limiting factor for their growth (Holford, 1997). Lately, it has been determined that plants associated with AM-fungi receive all of their phosphorus by the fungal symbiotic partner (Smith *et al.*, 2003).

The arbuscular mycorrhiza is an ancient mutualistic symbiosis originated more than 400 million years ago. In the 1990s, fossil records demonstrated that coevolutive strategies early in the evolution of plants improved their living conditions and let them adapt to a new living space on land (Remy *et al.*, 1994).

Because of the ancient origin of the symbiosis, conserved key mechanisms in molecular regulation are expected. So far, the physiology of this interaction is well characterized, but the understanding of the molecular regulation driving the AM-symbiosis is limited. To gain detailed insights into the processes of AM-symbioses is a big challenge for many researches all over the world.

In the last few years, continuative sequencing projects for model organisms have been established and supply a wide variety of possibilities in molecular genetics. For the model legume *Medicago truncatula* a public database is available providing more than 200,000 ESTs (www.medicago.org; www.genome.ou.edu/medicago.html). A new molecular era is initiated offering a chance to unravel the regulation and development of an AM-symbiosis in different approaches.

Actually, several differentially AM-regulated genes which are not expressed during other symbiotic interactions or after infection with plant pathogens have been identified by different *in silico* and experimental approaches (Frenzel *et al.*, 2005; Salzer *et al.*, 2000;

Wulf *et al.*, 2003). Recently, after the identification of AM-specifically induced genes, several genes have been characterized further. Mycorrhiza-specific expression patterns have been demonstrated, e.g. for a phosphate transporter gene (Harrison *et al.*, 2002), a glutathione-S-transferase (Doll *et al.*, submitted; Wulf *et al.*, 2003), lectin-like genes (Frenzel *et al.*, 2005; Frenzel *et al.*, submitted) and a trypsin inhibitor (Grunwald *et al.*, 2004).

Up to now, there is little knowledge about the functional role of these genes during the interaction. Therefore, it plays an important role in the comprehension of plant-microbe interactions and the underlying processes to understand the function of AM-specifically regulated genes.

Several reverse genetic techniques have been used to study the function of target genes. Recent studies have shown that the formation of double stranded RNA (dsRNA) can initiate mRNA degradation of the corresponding sequence (Meister and Tuschl, 2004). First proof of the involvement of dsRNA mediating gene silencing was giving by the discovery in *Caenorhabditis elegans* (Fire *et al.*, 1998; Fortunato and Fraser, 2005). This phenomenon was named RNA interference (RNAi) and since then it has become clear that this gene silencing phenomina taking place at the post-transcriptional level occurs in many organisms. Similar effects have already been described before in the 1990s. A specific degradation of homologous RNAs was first observed after introduction of an extra copy of an endogenous gene into plants (Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990). The phenomenon was originally called co-suppression, because RNAs encoded by both transgenes and homologous endogenous genes were degraded. The mechanism of PTGS plays an important role during plant defense against viruses (van der Krol *et al.*, 1990). The phenomenon of post-transcriptional gene silencing (PTGS) was also observed in fungi (*Neurospora crassa*) and was named quelling (Cogni *et al.*, 1996) and as gene silencing with antisense RNA (Fire *et al.*, 1991). The PTGS mechanism results in the specific degradation of endogenous RNA in the presence of homologous dsRNA either locally injected or transcribed from an inverted-repeat transgene (Baulcombe, 2005; Tavernarakis *et al.*, 2000). Although the corresponding mechanism is not fully understood, it seems that the underlying molecular mechanisms are highly conserved.

Nowadays the PTGS phenomenon is widely used as a tool to reduce or silence the expression of a target gene. In plants, several different approaches are used generating stable transgenic plants that express RNAs capable of forming a double-stranded

hairpin structure (Chuang and Meyerowitz, 2000; Waterhouse *et al.*, 1998; Wesley *et al.*, 2001). In *Nicotiana benthamiana* RNAi has been applied using *Agrobacterium tumefaciens* mediated transient expression (Johansen and Carrington, 2001) and, in cereals, biolistic delivery of dsRNA to leaf epidermal cells by particle bombardment resulted in interference with the function of endogenous genes at the single cell level (Schweizer *et al.*, 2000).

For organisms, such as *M. truncatula*, for which genome sequencing projects exist, PTGS might be one tool to study gene function at different conditions. The effectiveness of PTGS for functional genomics has already been demonstrated in the nematode *C. elegans* (Fire *et al.*, 1998), but has yet to be fully utilized in plants.

Up to now, only primary transformed roots using *Agrobacterium rhizogenes* mediated transformation were used to show silencing effects in the model legume *M. truncatula* (Limpens *et al.*, 2003) and *Lotus japonicus* (Kumagai and Kouchi, 2003). So far, no stable transgenic PTGS plants were reported to unravel functional analysis in the AM-symbiosis.

We have selected two candidate genes for a PTGS approach. Both genes are specifically expressed in arbuscular mycorrhiza symbiosis and had been identified in a SSH cDNA library enriched by mycorrhiza-specifically induced genes (Wulf *et al.*, 2003). *S*o far, the functional role of these genes has not been determined. *MtGlp*1 is a gene with high similarity to germin-like proteins. *In situ* hybridization could localize the *MtGlp*1 transcripts in arbuscule-containing cells (Doll *et al.*, 2003). And the second gene, *MtGst*1, a gluthatione-S-transferase gene in *M. truncatula* is specifically induced in the AM-symbiosis (Wulf *et al.*, 2003). The promoter of *MtGst*1 could have been identified and gene regulation analyses revealed mycorrhiza-specific binding motifs for transcription factors (Doll *et al.,* submitted). To understand the role of AM-specific genes, we have chosen a PTGS approach in whole transgenic plants. As an alternative to mutant screening or transgenic root tissue, stable transgenic plants may facilitate the analysis of gene functions under different conditions and in various genetic backgrounds.

Chapter 5

Materials and Methods

Plant growth and cultivation

Medicago truncatula cv. Jemalong 2HA seeds (Rose *et al.*, 1999) were sterilized for 10 min in sulfuric acid, washed in water and finally treated with 6% NaOCl (v/v) for 5 min. Seeds were germinated on water agar, first in the dark for three days and then at daylight for two days at RT. The 5-day-old seedlings were cultivated further on Shb10 media according to the protocol of Chabaud *et al.* (2003) for another 3 weeks. Under constant conditions (220 $\mu Em^{-2}s^{-1}$ for 16h; 22°C, 65% humidity), plants were cultivated *in vitro*.

Cloning *MtGlp*1 and *MtGst*1 cDNA-fragments into the PTGS vector

The GATEWAY cloning technique (Invitrogen, Karlsruhe, Germany) simplified the cloning of the binary vector pFGC5941. This PTGS vector contains a kanamycin resistance gene for bacterial selection and a bar gene to select transgenic plants with PPT in the media. Modified primers with attachment B sites according to the manufacturer were used. A ~600 bp long cDNA sequence of *MtGlp*1 was amplified using specific primers (5´ to 3´: forward AGT TGT TCT CTC ACT CAT CAT TTC CAC and reverse GCA CCC ATC ACC AAG CCT TAC TAA) and a ~350 bp long cDNA sequence of *MtGst*1 were achieved with following primers (5´to 3´: forward AGC CAT TCC TCA TTC TGC ATG GTT and reverse GCT ATG AGA CAC ATA AGC ATA TTC). *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) was transformed with the corresponding binary vectors via electroporation.

Agrobacterium tumefaciens mediated transformation of *M. truncatula*

Transformation of *M. truncatula* with these PTGS-mediating vectors has been done via somatic embryogenesis. According to Chabaud *et al.* (2003), we transformed leaflets of 3 week old 2HA *M. truncatula* plants with the *A. tumefaciens* strain EHA105. The *A. tumefaciens* strain containing the corresponding binary vectors was grown ON in liquid YEP media containing 50 µg/ml kanamycin and 5 µg/ml rifampicin. The ON-culture was

centrifuged and resuspended in YEP media. Leaflets were scratched with forceps under sterile conditions. Afterwards they were incubated in an *A. tumefaciens* solution with an OD_{260} of 0.1. Transformed leaflets were set on callus-inducing medium and were incubated one week in the dark at 24°C. Starting one week after transformation, 3 mg/l PPT was given to the medium to select for transgenic tissue. On appropriate media, as mentioned by Chabaud *et al.* (2003), callus-, embryo- and shoot development was provoked under constant conditions (220 $\mu Em^{-2}s^{-1}$ for 16h; 22°C, 65% humidity). Root development of the explants, deriving from these transformation approaches, was induced with additional auxin supplement (1µM IAB) in the final media Shb10.

Cultivation of transgenic plants

After rooting, transgenic plants of the T0-generation were transferred into the greenhouse under constant conditions at 18°C. Transgenic plants were planted into pots (Ø 24 cm) and were fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1950).

Inoculation with *Glomus intraradices*

For inoculation with the mycorrhiza fungus *G. intraradices,* a commercially availably inoculum by Biorize Sarl (Dijon, France) was used. Plants were inoculated and harvested as described earlier by Doll *et al.* (2003). For the calculation of mycorrhizal colonization intensity according to Trouvelot *et al.* (1986), incidentally selected roots were stained (Vierheilig *et al.*, 1998).

Leaf paint assay

Leaf paint assays were performed using the total herbicide BASTA® on *M. truncatula* leaves. A concentration of 600 mg/l glufosinate ammonium was required according to the protocol of Datta *et al.* (1992). Plants were evaluated 4 days after application.

RNA extraction

The LiCl method of Franken and Gnädinger (1994) was utilized to extract total RNA out

of *M. truncatula* roots.

Genomic DNA extraction

Leaf material of *M. truncatula* was used for the genomic DNA extraction according to Dellaporta *et al.* (1983).

Primer design

To exclude the false amplification of *MtGlp*1 and *MtGst*1 in transgenic plants, the primers have been designed to bind to the 5´ end of the cDNA sequence, but still outside of the region used to construct the PTGS vector. For *MtGlp*1 a ~90 bp sequence at the utter 5´ end was used (5´ to 3´: forward CCA ACA ACA TCT TCA AAG and reverse CAA GGT AGA GGA AGA CAT G). The *MtGst*1 transcript was amplified using specific primers at the 5´ end of the cDNA sequence producing a ~350 bp sequence (5´ to 3´: forward CTC CCT TTG ACA TGT TAG C and reverse CCA AAT GCT CCT CCC AGT GTC). These primers were used to create the DIG-labeled cDNA probes for hybridization techniques. Furthermore, they were required for qRT-PCR amplifications.

SMART cDNA synthesis and virtual Northern hybridization

Five µg RNA were used to produce SMART cDNA with the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, USA). After amplification, 1 µg of SMART cDNA was separated on 1% (w/v) agarose gels, blotted on a nylon membrane and hybridized to Digoxygenin labeled cDNA fragments. Digoxygenin labeled cDNA probes were synthesized by amplification of insert sequences using the Digoxygenin-labeling mix (Roche Diagnostics Corporation, Mannheim, Germany). Signals were detected according to the protocol of the Digoxygenin supplier (Roche Diagnostics Corporation, Mannheim, Germany).

Quantitative real-time PCR

Quantitative real-time PCR was carried out using the Opticon real-time cycler (MJ

Research, Waltham, MA, USA) and QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Total RNA (100 ng) was added to each amplification reaction. Reverse transcription was initiated in the first 30 min at 50°C, followed by the PCR program with an initial denaturation of 15 min by 95°C, amplification and quantification program was repeated 45 times (94°C for 15s, 45°C for 30s, 72°C for 30 s, with a single fluorescence measurement), and the melting curve program (40 to 95°C, with fluorescence read every 1°C).

Southern hybridization

Genomic DNA (20-40 µg) were digested with 6 units of appropriate restriction enzymes for 4 hours and ON with additionally 6 units. The digested DNA was separated on a 1.2% (w/v) agarose gel at 15 V. The gel was incubated for 10 min in 0.25 M HCl, 15 min in denaturing solution and 15 min in neutralization solution. A capillary blot transferred the DNA to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The hybridization and detection was carried out with the DIG system by Roche Diagnostics (Mannheim, Germany).

Chapter 5

Results & Discussion

Functional genomics: a great challenge to enlighten the arbuscular mycorrhiza interaction

Recent studies have identified several genes that are involved in an AM-symbiosis. A subtractive suppressive hybridization (SSH) technique lead to a cDNA library enriched by mycorrhiza-specifically expressed genes of *Medicago truncatula* (Wulf *et al.*, 2003). Starting from such a wide range of newly identified genes with specific AM-regulation, various molecular analyses were carried out. Gene structure and regulation analyses already lead to new knowledge of molecular mechanisms driving an AM-symbiosis. For example, it has been shown that the specific gene regulation of the trypsin inhibitor, *MtTi*1, is associated with arbuscule development (Grunwald *et al.*, 2004). *In situ* hybridization showed a specific expression of a mycorrhiza-specific germin-like protein, *MtGlp*1, in arbuscule-containing cells of *Glomus intraradices* colonized roots of *M. truncatula* and *Lotus japonicus*, indicating that the gene regulation is conserved during AM-colonization of roots of different plant species (Doll *et al.*, 2003). Mycorrhiza-specific expression patterns have also be demonstrated for a glutathione-S-transferase. The identification of the *MtGst*1 promoter (Wulf *et al.*, 2003) and elements mediating a mycorrhiza-specific transcriptional induction (Doll *et al.*, 2005 submitted) provide the starting point for the identification of corresponding mycorrhiza-specific transcription factors.

One great challenge in the near future will be to clear up the regulation of AM-specifically induced genes and the functional role of the corresponding proteins in this interaction.

PTGS as a tool to reduce the expression of a target gene

Since the post-transcriptional gene silencing (PTGS) mechanism has been found in a wide variety of organisms (Baulcombe, 2004), PTGS has become a widely used tool to reduce the expression of a target gene. We have set up an approach for two AM-specific genes of *M. truncatula*. The two candidate genes are both specifically expressed during arbuscular mycorrhiza symbiosis and represent a glutathione-S-

transferase like gene, *MtGst*1 (Doll *et al.*, 2005 submitted; Wulf *et al.*, 2003) and a gene with similarity to germin-like proteins, *MtGlp*1 (Doll *et al.*, 2003). As mentioned before, they both derive from the SSH library enriched by AM-specifically induced genes (Wulf *et al.*, 2003).

Currently, the PTGS phenomenon is widely used as a tool to reduce the expression of a target gene and thus study gene functions. For this purpose, we constructed binary vectors which contained downstream of a 35S-promoter cDNA fragments of *MtGlp*1 and *MtGst*1, in sense and antisense orientation flanking an intron sequence. The arrangement of antisense and sense sequence with the intron functioning as hair-pin structure will provide the production of dsRNAs. Nowadays, it is known that dsRNA can initiate mRNA degradation of the corresponding target sequence and therefore dsRNA builds the starting point of PTGS (Tavernarakis *et al.*, 2000). Transformation of *M. truncatula* with PTGS-mediating vectors has been done via somatic embryogenesis (Fig. 1).

Transgenic *Medicago truncatula* plants via somatic embryogenesis

So far, the expendable process of somatic embryogenesis revealed in a number of transgenic plants regenerated for both vector constructs of *MtGlp*1 and *MtGst*1 (Fig. 1). Yet, a foremost selection of the putative PTGS lines for *MtGlp*1 and *MtGst*1 occurred while in *in vitro* culture on media containing PPT for selection of transgenic tissue. With a successful transfer of the T-DNA into the plant genome, the putative PTGS plants could transcribe the bar gene and hence they can stand constant addition of PPT in the media. In contrast, non-transgenic plants died on media containing PPT after 3 days. Moreover, the transgenicity and a successfully integration of the T-DNA into the *M. truncatula* genome of these putative PTGS plants was proven by different approaches. All analyses were carried out with the T0-generation. So far, all putative PTGS lines for *MtGlp*1 and *MtGst*1 are transferred into the greenhouse to produce seeds of the T0-generation. Continuing transgenic generations will provide more knowledge about the stability and inheritance of the T-DNA. Leaf paint assays using BASTA® application affirmed the transgenicity of the putative PTGS lines. Exclusively leaves of transgenic plants remained invariably uninfluenced after spraying them with a BASTA® solution which was toxic and lethal for leaves of non-transgenic plants (Fig. 2A). Furthermore, the transfer of the T-DNA into the genome of *M. truncatula* was checked by PCR

approaches. A strong amplification of the Chsa-intron, flanking sense and antisense *MtGlp*1 or *MtGst*1 respectively, and the bar gene was achieved in all putative PTGS lines analyzed and was not detectable in the genome of non-transgenic plants (Fig. 2B). Since PCR results can not give evidence of the amount of integrated T-DNA copies, Southern hybridizations were performed (Fig. 2C). Southern hybridization analyses are nowadays the standard method used to detect the copy number of integrated genes and additively to identify variably different transgenic lines based on the integration patterns (Southern, 1975).

Gene silencing effects of *MtGlp*1- and *MtGst*1-PTGS-lines

Beyond, selected PTGS lines were layered by cuttings for large scale operations under mycorrhizal environmental conditions. The virtual Northern hybridization technique is a sensitive method, which we used to study the silencing effect at the transcript level. Using a SMART cDNA of mycorrhizal and non-infected putative PTGS plants in contrast to non-transgenic plants, silencing effects were obvious (Fig. 3). Using cDNA-specific probes of *MtGlp*1 and *MtGst*1 respectively, the corresponding transcript could be detected in mycorrhizal non-transgenic roots, and nowhere else. Since no transcripts of the corresponding AM-specific genes were detectable in the putative PTGS lines analyzed, we can speak about a knock down effect.

However, to make a quantified predication of down regulation effects, we established a more sensitive quantitative real-time PCR approach. Expression levels are relative to the level of the constitutively expressed translation elongation factor α, *MtEf*1. The mycorrhiza-specific phosphate transporter, *MtPT*4, was used as a marker gene for mycorrhiza development. We could detect silencing effects in several putative PTGS lines in different dimension (Fig. 4). For account of *MtGlp*1, the qRT-PCR revealed in several silenced lines. In Fig. 4, two representative GLP-PTGS lines are shown with a strong reduction of the *MtGlp*1 transcripts. In addition to putative GST-PTGS-lines showing a reduced *MtGst*1 transcription level, we could detect 2 putative lines with a similar transcription level as in mycorrhizal non-transgenic plants. The *MtGst*1 transcription level is equally high compared with mycorrhizal non-transgenic plants (e.g. for GST-PTGS-3, Fig. 4). Especially these two lines showing no reduction of *MtGst*1 have to be notably examined with further Southern analyses. It can be speculated that they might contain multi-copy numbers of the T-DNA abolishing the silencing effect.

Chapter 5

Correlation between silencing effects and entire expression patterns in mycorrhizal transgenic *M. truncatula* roots

So far, putative transgenic plants containing PTGS constructs did not show striking detectable phenotypic changes after inoculation with AM-fungi. On the one hand, these transcripts might give an unspecific response of *MtGlp*1 and *MtGst*1 respectively against *G. intraradices*. On the other hand, the functional role of *MtGlp*1 or *MtGst*1 in the mycorrhiza interaction was substituted by other genes involved in the symbiosis. Still, distinct and intensive phenotypic examinations are required to complete these analyses.

To study the influence of PTGS plants on the transcription profiles of other genes, we will consider the microarray technology available for approximately 16,000 *M. truncatula* genes on an Mt16KOL microarray. A microarray provides an enormous amount of information on the relationship between genes giving variable expression patterns in different tissue (Hohnjec *et al.*, 2005; Küster *et al.*, 2004). We expect to get new informative data provided by the microarray technology to project onto our PTGS approach for functional analyses. Since these analyses will follow in the near future, thus leaves us to speculate about the function of these AM-specific genes.

We have to consider what is known so far about germin-like proteins and how can acquainted functions of other plant GLPs be transferred to the single characterized AM-specific MtGLP1. This does also apply to the unique AM-specific glutathione-S-transferase of *M. truncatula* as well. Therefore, an overlook above GLPs and GSTs will be given in the general discussion (Chapter 6). First speculations of the functional role in the mycorrhiza symbiosis will be accomplished. Eventually, these speculations have to be confirmed with more detailed information by microarray analyses.

Figure Legends

Figure 1: somatic embryogenesis generated transgenic *Medicago truncatula* plants

Agrobacterium tumefaciens mediated transformation of *M. truncatula.* Different phases of the somatic embryogenesis process are shown: transformed leaves after incubation with the *A. tumefaciens* solution (1, 2), generated calli tissue (3), embryogenic tissue (4), plantlets *in vitro* (5) and T0-generation under greenhouse conditions (6).

Figure 2: proof of T-DNA transfer into the *M. truncatula* genome of putative PTGS lines

A leaf paint assay. 600 mg/l BASTA® solution was sprayed on leaves of non-transgenic *M. truncatula* plants (1) and transgenic putative PTGS plants of the T0-generation (2: e.g. GLP-PTGS-25). **B** PCR analyses with genomic DNA. The insertion of the T-DNA was proven by the amplification of bar (*1* for GLP-PTGS-line 25, *2* for GST-PTGS-line 13) and the Chsa-intron (*5* for GLP-PTGS-line 25, *6* for GST-PTGS-line 13) with appropriate primer pairs in the transgenic plants. There was no amplification in non-transgenic plants neither for bar (*3*) nor for Chsa-intron (*4*) **C** Southern hybridization with DIG-labeled probe of the bar gene. Using 40 µg of non-transgenic (1) and putative GST-PTGS-13 transgenic plant (2). DIG-labeled marker II is indicated with M.

Figure 3: RNA accumulation in PTGS-GLP-25 and PTGS-GST-13 roots

Virtual northern hybridization of two putative PTGS lines for *MtGlp*1 and *MtGst*1. SMART cDNA was synthesized using 5 µg total RNA from roots. Non-transgenic *M. truncatula* plants (3, 4, 7, 8). The transgenic putative PTGS lines GLP-PTGS-25 (1, 2) and GST-PTGS-13 (5, 6) are presented here. Plants were colonized by *Glomus intraradices* (1, 3, 5, 7) for 3 weeks. Non-inoculated roots (2, 4, 6, 8) were harvested after 3 weeks as well.

Figure 4: Graphical representation of comparative expression levels of putative PTGS lines

Template RNA was extracted of non-infected (-) and mycorrhizal roots (+) of transgenic and non-transgenic *M. truncatula* plants (wild type roots). As example, 3 putative PTGS

lines are presented: GLP-PTGS-25; GLP-PTGS-74; GST-PTGS-3; GST-PTGS-13. Amplification of *MtPt*4 (PT) was used as a marker gene for the mycorrhization intensity. Amplification of *MtGlp*1 (GLP) and *MtGst*1 (GST) were applied with primers at the 5´end of the corresponding cDNA. Ct-values were determined by quantitative RT-PCR and were used to calculate comparative expression levels ($2^{-\Delta Ct}$). Expression levels are relative to the level of *MtEf*1 expression, which was constant in all RNA samples used.

Figure 1

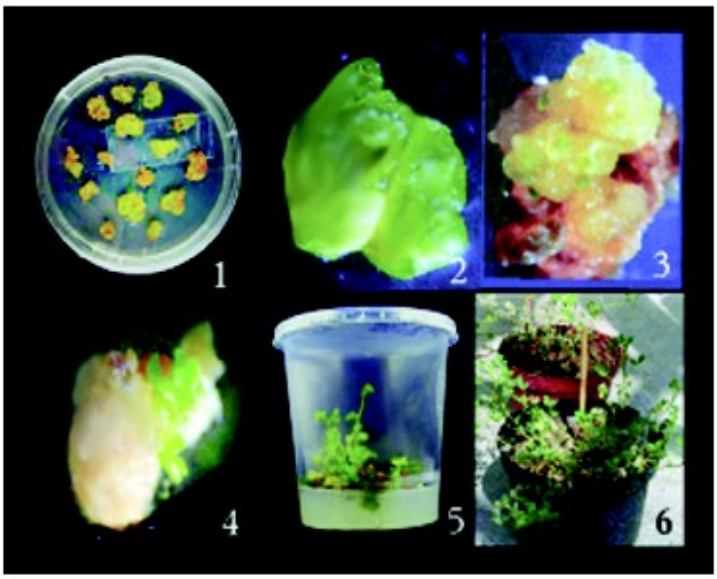


Figure 2

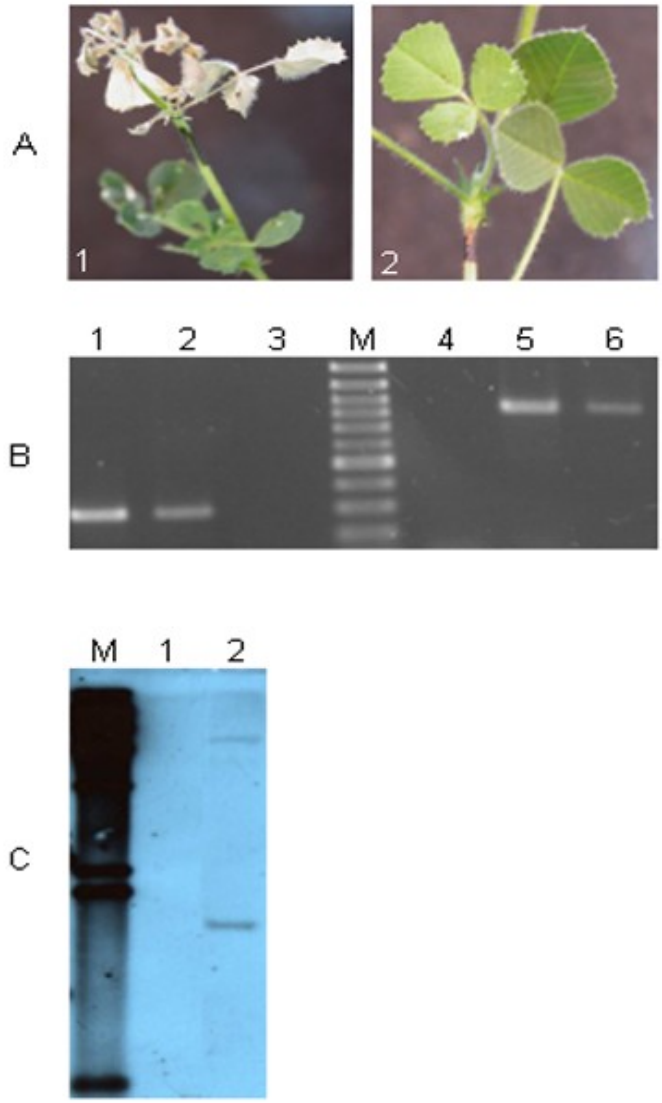


Figure 3

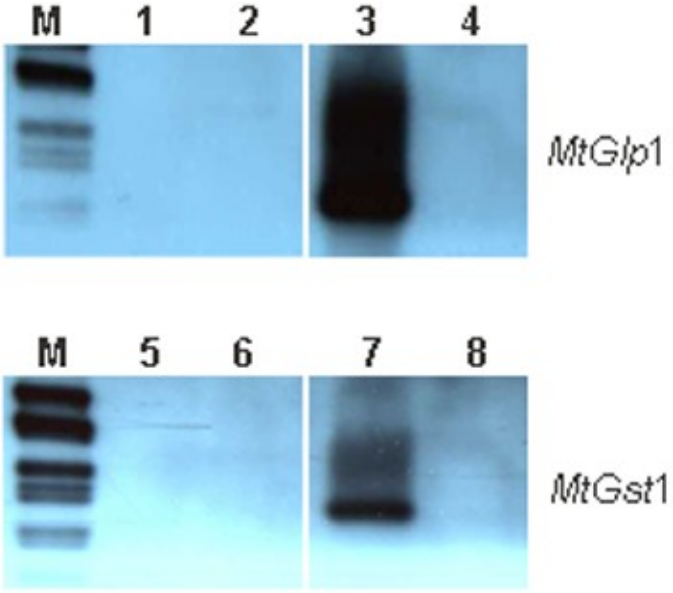
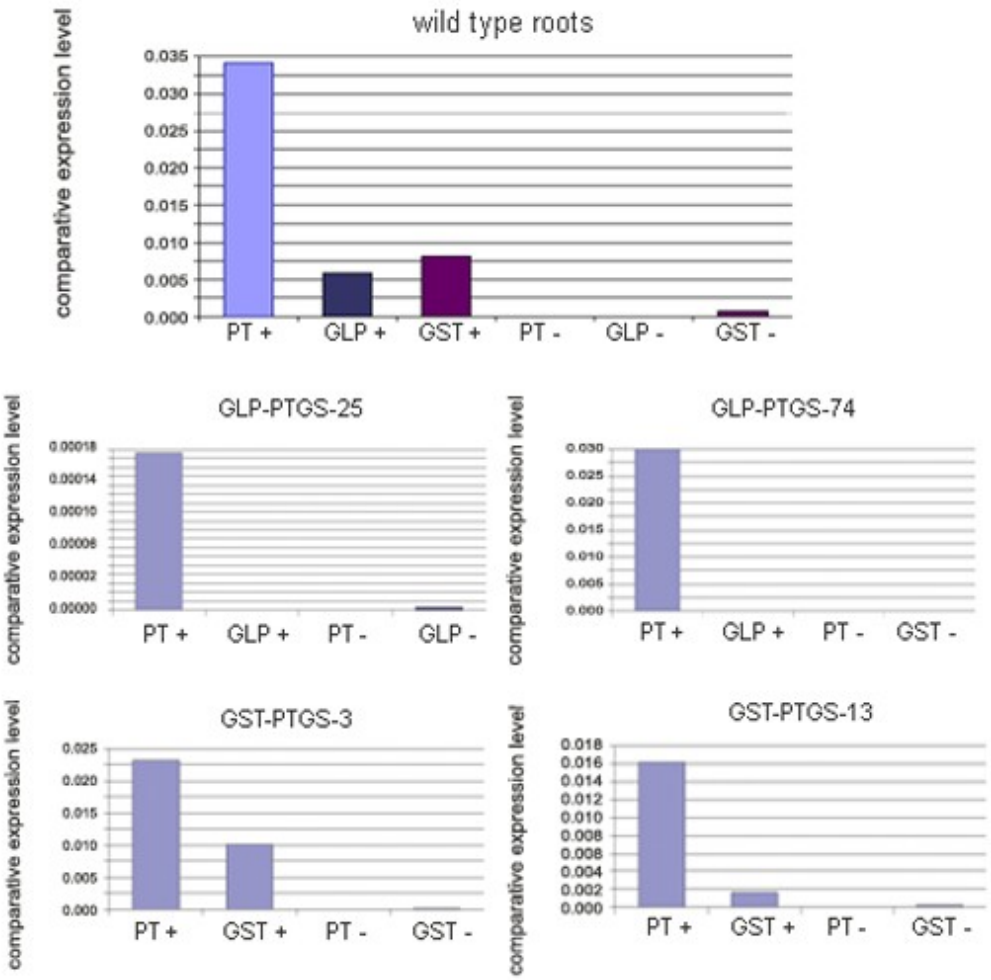


Figure 4


wild type roots
comparative expression level
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
PT +
GLP +
GST +
PT -
GLP -
GST -
GLP-PTGS-25
comparative expression level
0.00018
0.00014
0.00010
0.00006
0.00002
0.00000
PT +
GLP +
PT -
GLP -
GLP-PTGS-74
comparative expression level
0.030
0.025
0.020
0.015
0.010
0.005
0.000
PT +
GLP +
PT -
GST -
GST-PTGS-3
comparative expression level
0.025
0.020
0.015
0.010
0.005
0.000
PT +
GST +
PT -
GST -
GST-PTGS-13
comparative expression level
0.018
0.016
0.014
0.012
0.010
0.008
0.008
0.004
0.002
0.000
PT +
GST +
PT -
GST -

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Chapter 6

Supplementary Discussion

Medicago truncatula a model plant to study molecular processes in arbuscular mycorrhiza interaction

Recently, transcriptome analyses in various model organisms have been applied for a comprehensive analysis of plant-microbe interactions (Colebatch *et al.*, 2002; Fedorova *et al.*, 2002; Journet *et al.*, 2002). We have chosen the model plant *M. truncatula* to study the molecular background of the arbuscular mycorrhiza symbiosis.

Since the 1990s, this legume plant has emerged as a widely accepted model to study plant and legume genomics (Barker *et al.*, 1990; Cook, 1999). *M. truncatula* has a relatively small and simple genome, but is phylogenetically related to the most important crop legumes such as pea, alfalfa, chick pea and faba bean. Genomic data obtained through research with the model plant *M. truncatula* allows a transfer of the corresponding information to other legumes.

An extended resource of sequence information has become available during the last few years. A sequencing project for the *M. truncatula* genome was initiated in 2001 (www.genome.ou.edu/medicago.html), but to date the project is not complete yet. However, *M. truncatula* is subject to large scale EST analysis (www.medicago.org, www.tigr.org/tdb/mtgi/) providing more than 200,000 ESTs available in public databases (Chapter 1).

The majority of available ESTs of *M. truncatula* arise from different non-subtractive cDNA libraries. However, there are several plant genes with low-level transcription, whose transcripts are not, or sparsely represented, in non-subtractive cDNA libraries. The subtractive suppressive hybridization (SSH) technique allows creating cDNA libraries enriched by differentially expressed genes (Diatchenko *et al.*, 1996). Therefore, a cDNA library of exclusively AM-induced genes of *M. truncatula* was created (Chapter 2).

With this SSH technique, several transcripts of our model plant were identified, showing strong RNA accumulation in mycorrhizal *M. truncatula* roots, which were not detectable

in sterile roots or in roots infected with *Sinorhizobium meliloti*. These were also not detectable after infection with the pathogen *Aphanomyces euteiches* neither in roots treated with additional phosphate fertilization. Observed transcriptional changes were confirmed by quantitative RT-PCR (Chapter 2).

The AM-specific induced sequences constituted the starting point for several further research projects. Consequently, *MtGim* 30 (Chapter 2) was annotated as trypsin inhibitor *MtTi*1 and it was demonstrated by Grunwald *et al.* (2004) that the gene activity of *MtTi*1 is clearly associated with arbuscule development.

Three different genes with similarities to plant lectins were identified (Chapter 2). In addition, Frenzel *et al.* (2005) could identify more AM-regulated lectins by *in silico* analysis. This novel family of AM-specific lectin genes was suggested to be involved in different transport and signaling processes. At least two lectin members were shown to play a role in arbuscule formation or functioning.

For other significant AM-specific genes, like a blue-copper binding protein, a nitrate transporter and a syringomycin-biosynthesis like protein (Chapter 2), detailed investigations were initiated and will be carried out in our working group.

In this study, the latest findings of two AM-regulated genes, representing most promising candidates of the SSH library (Chapter 2), will be considered.

A highly conserved arbuscule specific germin-like protein

The deduced amino acid sequence of one of the AM-specifically induced genes, *MtGlp*1, showed high similarity to germin-like proteins (GLPs) (Chapter 3). GLPs are ubiquitously distributed among land plants and constitute a large and functionally diverse family of plant proteins (Bernier and Berna, 2001; Druka *et al.*, 2002; Membre *et al.*, 2000) participating in many processes that are important for plant development (Aubrey *et al.*, 2003; Lane *et al.*, 1993) and defense (Johnk *et al.*, 2005; Park *et al.*, 2004). While true germins are mostly expressed during germination and under stress conditions, germin-like proteins are expressed at all developmental stages and some of them are influenced by abiotic as well as biotic stress conditions (Bernier and Berna, 2001, Dani *et al.*, 2005; Vallelian-Bindschedler *et al.*, 1998). In contrast, *MtGlp*1 seems to be involved exclusively during an AM-interaction. By various approaches and comparisons of distinct cDNA libraries derived from AM-tissues, the mycorrhiza-specificity had been proven (Chapter 3).

Germins and GLPs are composed of three highly conserved oligopeptides that are called germin boxes A, B and C (Bernier and Berna, 2001). The boxes B and C, called the cupin domains, are present in the amino acid sequence of MtGLP1 (Chapter 3). The highly conserved box A appeared to not be conserved in the MtGLP1 sequence, which suggests that MtGLP1 can not be classified into one of the three postulated subfamilies of *Arabidopsis thaliana* (Carter *et al.*, 1998).

*MtGlp*1 is not the only mycorrhiza-specific gene that could not be associated with corresponding gene family members. Recently, it has been reported that *MtPT*4, a mycorrhiza-specific phosphate transporter, is significantly different from phosphate transporters of *M. truncatula* as well as of *A. thaliana*, rice or potato. *MtPt*4 does not show more than approximately 60% similarity to the other phosphate transporters which are all not involved in an AM-symbiosis (Harrison *et al.*, 2002). *MtPt*4 is currently accepted and used as a marker gene for a functional AM-symbiosis. Since phosphate uptake plays a major role in the nutrient exchange taking place in this interaction, it is not surprising that 2.97% of the 1,805 SSH ESTs matched with the corresponding sequence of *MtPt*4 (Chapter 2).

A large number of GLP genes have been discovered in higher plants. For example, in barley, at least 14 GLP genes have been identified (Druka *et al.*, 2002). In rice, at least eight different *Glp* sequences were identified (Membre and Bernier, 1998). However, the best characterized GLP family is in *A. thaliana*, where 27 individual family members were identified and classified into 3 subfamilies (Carter *et al.*, 1998). We included all available TC sequences of *M. truncatula* in addition to the *A. thaliana* GLP sequences in a multiple sequence alignment. Most of these GLP-encoding genes of *Medicago* grouped with the three *Arabidopsis* GLP subfamilies. Certainly, MtGLP1 - the only mycorrhiza-specific GLP in this analysis - was significantly different to all other genes analyzed. This is not surprising since *A. thaliana* is not capable of forming plant-microbe interactions, such as the AM-symbiosis. Therefore, we suggest that *MtGlp*1 represents a member of a new subfamily of plant GLPs (Chapter 3).

In situ hybridization detected *MtGlp*1 transcripts in arbuscule-containing root cortex cells. Additionally, the putative orthologue of *MtGlp*1 could have been localized in a second model legume, *Lotus japonicus* (Chapter 3). This speaks for an underlying conserved mechanism in the specific AM-regulation of GLP family members in different plant species. Although *L. japonicus* is currently a vast studied model legume of which also sequencing projects and a selection of sequence information exist, the orthologue

of *MtGlp*1 has not been discovered and characterized so far.

Since *MtGlp*1 transcripts were also found in cDNA libraries of mycorrhizal roots infected with other AM-fungi than *Glomus intraradices*, this gene can be assumed to represent an AM-specific marker gene.

With all this new and most informative information of *MtGlp*1 first investigations in the enlightenment of molecular processes driving an arbuscular mycorrhiza were exposed. Determining the localization of *MtGlp*1 and its exclusiveness in the GLP gene family compared to GLP members of *A. thaliana* (Chapter 3), the function of the plant GLPs is still obscure.

Therefore, we attempted a knock-down approach by post-transcriptional gene silencing (PTGS) to unravel the function of *MtGlp*1 in an AM-interaction (Chapter 6). Transformation of *M. truncatula* with PTGS-mediating vectors has been done via somatic embryogenesis. The arrangement of antisense and sense sequence in the constructed binary PTGS vectors provided the production of dsRNAs which are required as starting material for the PTGS mechanism (Meister and Tuschl, 2004). So far, a substantial number of transgenic plants have been regenerated. First analyses of transgenic PTGS plants, representing the T0-generation, were done giving proof about the integration of the T-DNA by leaf paint assays using BASTA® application, PCR approaches and Southern hybridizations (Chapter 6).

However, the silencing effect mediated with dsRNA has been shown to be more efficient as by antisense or sense approaches (Levin *et al.*, 2000). In most cases, a gene inactivation could not be reduced to 100%. In case of *MtGlp*1, silencing effects could have been detected for several putative PTGS lines analyzed by qRT-PCR approaches. After PCR amplification of the transcript of *MtGlp*1 could still be detected in the putative PTGS lines. However, in quantitative real-time PCR approaches, which constitute a more sensitive method, we could detect silencing effects in several putative PTGS lines in different dimensions (Chapter 6).

To study the influence of the reduction of an AM-specific gene to the transcription profiles of other genes, we will consult the microarray technology available for approximately 16,000 *M. truncatula* genes on an Mt16KOL microarray. Microarrays provide an amount of information on the relationship between genes giving variable expression patterns in different tissue. Microarray analysis have to follow this work. Since we do not have any data, up to now, we can only presume a function for *MtGlp*1.

Hitherto, GLPs have been found to possess a number of functions, ranging from

antioxidative metabolism to signal reception, transduction and structural roles (Bernier and Berna, 2001). Several true Germins of *Gramineae* possess oxalate oxidase (OxO) activity. Further, superoxide dismutases (SOD) activity could have been determined for some germin-like proteins (Carter and Thornburg, 2000; Christensen *et al.*, 2004; Kukavica *et al.*, 2005; Segarra *et al.*, 2003; Yamahara *et al.*, 1999). Up to now, no enzyme activity for other GLPs of dicotyledonous plants could be exposed (Carter and Thornburg, 2000). Therefore, we do not expect such an enzymatic role for *MtGlp*1. Various GLPs are involved in processes in plants against pathogenic attack (Johnk *et al.*, 2005; Vallelian-Bindschedler *et al.*, 1998). Since some of the molecular events taking place while forming appressoria in fungal pathogens and AM-fungi seems to correlate, we can assume that MtGLP1 has a similar role as GLPs known in defense processes. A correlation with the reorganization of cell wall complexes after pathogenic attack, have been demonstrated for GLPs of barley (Wei *et al.*, 1998). Park *et al.* (2004) assumed for a GLP of *Capsicum annuum*, CaGLP1, a participation in important aspects of cell wall remodeling. That can be supported by the description of the subcellular location and biochemical properties of GLPs from *A. thaliana* (Membre *et al.*, 2000). For *MtGlp*1, we presume a functional role in the construction and development of arbuscules, since *MtGlp*1 transcripts were exclusively localized in arbuscule-containing cells (Chapter 3). But still the putative function of *MtGlp*1 is very speculative.

Functional genomics for an AM-specific glutathione-S-transferase

Another AM-specifically induced transcript of the SSH library showed high similarity to plant glutathione-S-transferases (GSTs). Therefore, we called the AM-specific gene of *M. truncatula, MtGst*1 (Chapter 2). GSTs were first discovered in the 1960s and up to now they have been found in animals, plants and fungi (Edwards and Dixon, 2000; Sheehan *et al.*, 2001; Wilce and Parker, 1994). Though GSTs represent a highly divergent family of well-studied genes, the functions of many GSTs are still barely understood. Mainly, GSTs seem to play an important role in a variety of enzymatic detoxification processes (Edwards *et al.* 2000; Timmerman, 1989) and in various stress conditions (Marrs, 1996). In potato, a PRP1-defense gene with high homology to glutathione-S-transferases was detected, which is induced after infection with different pathogens and with AM-fungi (Strittmatter *et al.*, 1996).

In contrast, *M. truncatulas MtGst*1 gene was clearly and exclusively induced in an AM-

symbiosis and not after pathogenic infection (Chapter 2). Moreover, the promoter of *VfLb*29 was found to be specifically active in arbuscule-containing cells of transgenic *Vicia faba* and *M. truncatula* roots infected with *Glomus intraradices* as well as in infected cells of the nitrogen-fixing zone of root nodules (Vieweg *et al.*, 2004).

Since several mycorrhiza induced genes turned out to be involved in both symbiotic interaction with Rhizobia and AM-fungi (Brechenmacher *et al.*, 2004; Journet *et al.*, 2001; Sanchez *et al.*, 2004; van Rhijn *et al.*, 1997; Vieweg *et al.*, 2004), we wanted to confirm the mycorrhiza-specificity of *MtGst*1 in addition to the RNA accumulation studies (Chapter 2/4).

We established a similar promoter reporter gene approach as described for p*MtGst*1 -836/+32 (Chapter 2). Both roots of *M. truncatula* and the non-legume plant *Nicotiana tabacum* were transformed with the promoter reporter constructs. Transgenic plants were infected with *Glomus intraradices*. Furthermore, *M. truncatula* was inoculated with *Sinorhizobium meliloti.* No transcription regulation of the reporter gene could be initiated in nodules, but an AM-specific activation of the reporter gene was achieved in mycorrhizal roots of *M. truncatula* as well as in *N. tabacum* (Chapter 4).

Since phytohormones are involved in different phases of the development and regulation of an AM-symbiosis (Barea and Azcón-Aguilar, 1982; Barker and Tagu, 2000; Wasternack and Hause, 2002), we analyzed the transcription of *MtGst*1 relating to different phytohormone treatments. No *MtGst*1 transcripts were detectable in *M. truncatula* roots cultivated with different phytohormones (Chapter 4).

These results enhance our assumption that the regulation of *MtGst*1 is utterly AM-specific which is not limited to legumes. Besides, the results indicate furthermore that conserved transcriptional activation and regulation mechanisms with similar pathways exist in plant kingdom.

To receive a better understanding of the molecular mechanisms responsible for an AM-specific transcriptional regulation, the promoter of *MtGst*1 was analyzed with further approaches. Electrophoretic Mobility Shift Assay (EMSA) analyses and promoter deletion studies were performed and finally joined with computational analyses. Putative binding sites for mycorrhiza-specific transcription factors could be exposed in the promoter sequence of *MtGst*1 (Chapter 4).

*MtGst*1 regulation seems not limited to arbuscule-containing cortex cells. The reporter gene activity as shown in present work was detected also in other parts of the mycorrhizal root cells (Chapter 2 and 4). A speculation of the function of *MtGst*1 is

therefore more complicated, because it seems to be not limited to special compartments. Besides, *MtGst*1 seems not to be induced in early stages of an AM-symbiosis, but is strongly expressed in a fully developed AM-interaction (Brechenmacher *et al.*, 2004; Frenzel *et al.*, in preparation).

PTGS seems to provide an effective way to unravel gene functions (Bakhetia *et al.*, 2005; Fire, 1999; Ivashuta *et al.*, 2005; Limpens *et al.*, 2003). PTGS has revolutionized the study of genes in *C. elegans* and a great many of genes were analyzed systematically in *C. elegans* (Fortunato and Fraser, 2005; Fraser *et al.*, 2000; Gonczy *et al.*, 2002). For organisms such as *M. truncatula* whose whole genome is going to be sequenced completely, the next challenge would be to understand the functions and the interplay of genes in the organism. For this purpose, PTGS could be the method of choice. In the present work the PTGS constructs were transformed into *M. truncatula* to study the function of *MtGst*1 in complete transformed plants. Stably transformed plants facilitate the analysis of the function of MtGST1 - and MtGLP1 as described earlier – under different conditions and in various genetic backgrounds. Several putative PTGS lines resulted from the transformation via somatic embryogenesis (Chapter 6). With qRT-PCR we could detect PTGS lines indicating a reduction of the *MtGst*1 transcription level. These lines provide the starting point to study the function of MtGST1.

Beside the detoxification of herbicides in plants, a growing number of non-detoxification functions have been correlated with GSTs. Glutathione-S-transferases are involved in secondary metabolism, in stress metabolism and signaling (Dixon *et al.*, 2002). GSTs are considered to play an essential role in the preservation of plants during environmental stresses and diseases. Several stress-inducible GSTs protect plants from oxidative injury (Cummins *et al.*, 1999; Roxas *et al.*, 1997). GSTs are also related to stress tolerance processes through a role in cell signaling (Loyall *et al.*, 2000). Considering the role of the AM-specific GST of our work, *MtGst*1 could be involved in defense responses to AM-fungi. However, it may also function in arbuscule senescence, since GSTs have been found with protective functions in pea (Dixit *et al.*, 2001). Additionally, a tomato GST gene exhibits to be induced in cell death processes (Hoeberichts *et al.*, 2001).

Chapter 6

Future prospects

Using modern molecular biological methods vast numbers of *M. truncatula* genes specifically regulated by an AM were obtained. A great deal of sequence information is available nowadays, coupled with the development of bioinformatic tools they provide new opportunities to identify further AM-specific genes.

The available *M. truncatula* library (*Medicago truncatula* Gene Index, MtGI) with more than 200,000 ESTs arising from different cDNA libraries of variable tissues builds the foundation for *in silico* Northern strategies. By means of this strategy, a set of rhizobia specific genes of *M. truncatula* (Fedorova *et al.*, 2002) and a series of AM-specific transcripts in *M. truncatula* (Frenzel *et al.*, 2005) were identified collateral. Two other mycorrhiza-specific GLP sequences were found by *in silico* analyses of Frenzel *et al. (*2005). These newly identified genes have not been characterized further. However, according to their high homology to *MtGlp*1 they are promising new research objects and should be analyzed, characterized and compared to the information available from *MtGlp*1 in near future. For the silencing of *MtGlp*1, these two other AM-specific GLPs could have a main effect and responsibility of replacing *MtGlp*1.

The data available from the whole genome sequencing project of *M. truncatula* will make research in many ways easier. E.g., cloning of promoter regions of different genes will be much simpler and faster and these promoter studies will give important information about the expression regulation during symbiotic interactions. Furthermore, investigations should focus on the proteins themselves and the correlating functions in order to obtain more detailed comprehension of the molecular mechanisms underlying the AM-symbiosis.

Our first results of gene silencing by PTGS approaches encourage us to use this method for functional analysis of AM-induced genes. Up to now, there is no report on using microarrays to study global gene-expression pattern changes caused by silencing a particular endogenous gene by PTGS. But with enormous information provided by the microarray technology, we have good prospects to study the PTGS phenomenon of silenced genes giving different transcription profiles of other genes influenced by this effect. Above all, it is important to bring together all the information collected by different functional genomic tools.

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Appendix

PUBLICATIONS

Wulf A, Manthey K, Doll J, Prick, AM, Linke B, Bekel T, Meyer F, Franken P, Kuester H, Krajinski F

Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*.

Mol Plant Microbe Interact 2003 Apr; 16 (4): 306-314.

Doll J, Hause B, Demchenko K, Pawlowski K, Krajinski F

A member of the germin-like protein family is a highly conserved mycorrhiza-specific induced gene.

Plant Cell Physiol 2003; 44 (11): 1208-1214.

Doll J, Wolff S, Tiller N, Krajinski F

Insights into the transcriptional regulation of *MtGst*1 - an AM-specific gene of *Medicago truncatula*.

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Doll J, Xu Y, Krajinski F

PTGS approach to unravel the function of *MtGlp*1 and *MtGst*1, two AM-specific *Medicago truncatula* genes.

in preparation

CONGRESSES

- 3. Schwerpunktskolloquium "Molekulare Grundlagen der Mykorrhiza-Symbiosen" - Bad Honnef, 10. - 11.1.2002

- 4. Schwerpunktskolloquium "Molekulare Grundlagen der Mykorrhiza-Symbiosen" - Wittenberg, 12. - 14.12.2002:

„A member of the germin-like protein family is a highly conserved mycorrhiza-specifically induced gene“

- Workshop „Transformation“ - Bielefeld, 8.9.2003:

„RNAi/PTGS-approaches in transgenic *Medicago truncatula* tissue“

- *Medicago*-Symposium – Toulouse-Labège, France, 12. - 14.11.2003

- Botaniker-Tagung - Braunschweig, 2004, 5. -10.9.2004:

„Mycorrhiza-specific transcriptional changes in *Medicago truncatula*“

- Model Legume Congress – Pacific Grove, USA, 5. - 9.6.2005:

„Functional analyses of *MtGst*1 and *MtGlp*1, two AM-specific genes of *Medicago truncatula*“

- 6. Schwerpunktskolloquium "Molekulare Grundlagen der Mykorrhiza-Symbiosen" - Neustadt, 31.10. und 1.11.2005:

„PTGS approach to unravel the function of *MtGlp*1 and *MtGst*1, two AM- specific *Medicago truncatula* genes”

CURRICULUM VITAE

Name: **Jasmin Doll**

Date of birth: 21 August 1976

Place of birth: Zell am Harmersbach, Germany

Nationality: German

Family status: Unmarried

UNIVERSITY EDUCATION

July 2002 -December 2005 Ph.D. student
Molecular genetics, University of Hannover

April 1999 - April 2002 Study lectures in „biology (diploma)“
University of Hannover, Germany
Final grade: diploma

October 1996 - March 1999 Basic lectures in „biology (diploma)“
University of Freiburg, Germany
Final grade: prediploma

EDUCATION

1987 -1996 Secondary school, Offenburg, Germany

1983 - 1987 Primary school, Unterharmersbach, Germany

INTERNATIONAL EXPERIENCE

August – October 1999 Practical training, university of Toronto, Canada

Declaration

I declare that the studies presented above were composed and carried out all by myself, using only the mentioned materials and methods.

Furthermore, I declare that the work presented in this PhD thesis or parts out of it have not been published elsewhere previously.

I hereby affirm the truth of this statement with my signature.

Erklärung

Ich versichere hiermit, dass die vorliegende Dissertation von mir selbständig verfasst und die benutzten Hilfsmittel, Methoden und Quellen vollständig angegeben wurden.

Außerdem erkläre ich, dass diese Promotionsarbeit oder Teile daraus noch nicht veröffentlicht wurde.

Die Richtigkeit meiner Angaben bestätige ich mit meiner Unterschrift.

Hannover, 19.12.2005 Jasmin Doll

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